THE ANTIRACHITIC ACTION OF COD LIVER OIL AND IRRADIATED ERGOSTEROL IN PARATHYROIDECTOMIZED AND THYMECTOMIZED RATS

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(Received for publication, July 15, 1930)

The purpose of this study has been to ascertain whether the antirachitic action of cod liver oil and of irradiated ergosterol is dependent upon the integrity of the parathyroid or thymus glands.

I. The Effect of Parathyroidectomy

Several investigators have studied the effect of these agents in experimental tetany of dogs. The observations have been somewhat conflicting. Whereas Brougher (1), Wade (2), and more recently, Jones, Rapoport and Hoden (3), and Demole and Christ (4), were able to mitigate the symptoms of tetany, to prolong life, and to bring about an elevation of blood calcium after parathyroidectomy, Urechiu and Popoviciu (5), and Greenwald and Gross (6), were unable to demonstrate such effects. Wade found that the preoperative administration of cod liver oil mitigated symptoms and prolonged life, but did not prevent a fall in the blood calcium. Hess, Weinstock and Rivkin (7) produced latent tetany in a monkey by a low calcium diet, with a fall of the blood calcium to about 6 mg. The administration of a large amount of irradiated ergosterol *per os* raised the blood calcium to 11 mg. After thyroparathyroidectomy, the blood calcium could not be raised above 7 mg. In numerous further experiments upon dogs and monkeys, they were not able to raise the level of the blood calcium above normal, although administration of irradiated ergosterol for some weeks before operation resulted in a less precipitous fall in blood calcium after extirpation of the parathyroids. These experiments suggested to the authors that "a vitamine may function at least to a certain extent, through the medium of the ductless glands." In more recent experiments (8), the same authors have found that with very large doses of irradiated ergosterol (400 to 800 times the therapeutic dose), hypercalcemia could be produced even in the absence of the parathyroids.

We have found no previous work bearing directly upon the subject of this study.

Methods

Litters of young hooded rats of from 25 to 40 gm. in weight were used. Parathyroidectomy was performed in the usual way under amytal anaesthesia. The completeness of the extirpation was established in each case by complete serial sections of the excised tissue. The rats were placed on the Steenbock low phosphorus rickets producing diet from the onset of the experiment. Cod liver oil (Mead Johnson's tested) was administered with a pipette. For curative experiments, 5 daily doses were given after 21 days on the rickets producing diet. Radiograms were taken at the beginning and end of the treatment, the rats being killed 5 days after the last dose. Irradiated ergosterol (Mead Johnson's concentrated preparation having a potency 10,000 times that of cod liver oil), was diluted with 10,000 parts of olive oil, and administered by pipette. The ribs and tibia in each rat were decalcified in Mueller's fluid and studied histologically.

TABLE I

Litter A

Rat No.	Parathyroidectomy	Days on diet	Weight	Treatment	Rickets
3	I/20/30. Complete	21	35–52	C.L.O. 4 gtt. daily	0
7	I/20/30. Complete	21	35–40	None	++++
8	I/20/30	27	62–72	C.L.O. 4 gtt. for last 5 days	++++ Healing +
9	Control	27	31–39	None	++++
10	Control	27	29–36	C.L.O. 4 gtt. for last 5 days	++++ Healing ++
11	Control	27	28–38	C.L.O. 4 gtt. for last 5 days	++++ Healing +

Consistent results were obtained in all the experiments. The experimental data can best be presented in Tables I to VI, which summarize the essential findings.

Result of Experiment Shown in Table I.—Complete prevention of rickets in parathyroidectomized Rat 3. Rickets in untreated parathyroidectomized Rat 7; and in unoperated Controls 9, 10 and 11. Positive curative effect of 4 drops of C.L.O. after 5 days in parathyroidectomized Rat 8 and in Controls 10 and 11.

Result of Experiment Shown in Table II.—Complete prevention was obtained in parathyroidectomized Rats 5 and 6 with 4 drops of C.L.O. daily. Marked healing occurred in parathyroidectomized Rats 2 and 4, and in control unoperated Rats 8 and 9 after 5 doses of 2 drops of C.L.O. Marked rickets without spontaneous healing in control unoperated Rat 7.

Serial sections of the neck organs of B-2, B-4, B-5 and B-6 showed no remains of parathyroid tissue at the site of extirpation, and absence of

TABLE II

Litter B

Rat No.	Parathyroidectomy	Days on diet	Weight	Treatment	Rickets
2	I/24/30. Complete	34	37–78	C.L.O. 2 gtt. 5 days	++++ Healing +++
4	I/24/30. Complete	34	52–62	C.L.O. 2 gtt. 5 days	++++ Healing +++
5	I/24/30. Complete	26	33–53	C.L.O. 4 gtt. daily	0
6	I/24/30. Complete	26	33–50	C.L.O. 4 gtt. daily	0
7	Control	22	30–36	None	++++
8	Control	34	28–46	C.L.O. 2 gtt. 5 days	++++ Healing +++
9	Control	34	32–50	C.L.O. 2 gtt. 5 days	++++ Healing +++

any accessory glands. The entire thymus was included in the series of B-2, B-4 and B-6, and particular search was made for intra-thymic rests of parathyroid tissue. None were found.

Result of Experiment Shown in Table III.—Incomplete prevention with 2 drops of C.L.O. daily in Rat 1; complete protection in Rat 2. Marked healing with 2 to 4 drops of C.L.O. after 5 doses in parathyroidectomized Rats 3 and 4, and in unoperated Controls 5 and 6. No spontaneous healing in unoperated rachitic control Rat 7.

The experiments in Table IV show that similar results were obtained with irradiated ergosterol.

Result of Experiment Shown in Table IV.—Complete prevention was obtained in parathyroidectomized Rats 1, 2 and 3 with a daily dose of 1 gtt. of Acterol* diluted to equivalent strength of 1 gtt. of C.L.O. Marked healing was obtained after 5 doses of 1 gtt. of the same preparation in parathyroidectomized Rats 4 and 6, and in control unoperated Rats 8 and 9. It was noted that a less severe rickets developed in the operated than in the control rats, probably due to the more rapid gain in weight in the latter group. Complete serial sections

TABLE III

Litter C

Rat No.	Parathyroidectomy	Days on diet	Weight	Treatment	Rickets
1	II/3/30. Complete	28	24–30	C.L.O. 2 gtt. daily	+
2	II/3/30. Complete	28	30–40	C.L.O. 2 gtt. daily	0
5	II/3/30. Complete	39	38–62	C.L.O. 2 gtt. for 5 days	++ Healing ++
4	II/3/30. Only one Ek in sections; series incomplete	39	30–59	C.L.O. 4 gtt. for 5 days	++ Healing +++
5	Control	39	22–39	C.L.O. 2 gtt. for 5 days	+++ Healing +++
6	Control	39	28–43	C.L.O. 4 gtt. for 5 days	++++ Healing +++
7	Control	28	28–42	None	+++

of the neck organs of Rat D-6, including thymus gland, showed no remains of parathyroid tissue or accessory glands.

The experiments shown in Tables I to IV may be briefly summarized. Complete prevention of rickets in young rats on a Steenbock low phosphorus diet was obtained with daily dosage of 2 to 4 drops of cod

* Acterol was the trade name of the Mead Johnson irradiated ergosterol preparation, until supplanted by the official U.S.P. designation of Viosterol.

liver oil in spite of complete extirpation of the parathyroids. In one rat (C-1) complete protection was not afforded by a daily dose of 2 drops. Six parathyroidectomized rats given curative doses of 2 to 4 drops of cod liver oil for 5 days, all showed distinct healing.

TABLE IV

Litter D

Steenbock Diet

Rat No.	Parathyroidectomy	Days on diet	Weight	Treatment	Rickets
1	II/11/30. Complete	22	26–44	Acterol (conc.) 1–10,000; 1 gtt.	0
2	II/11/30. Complete	22	23–36	Acterol (conc.) 1–10,000; 1 gtt.	0
3	II/11/30. Complete	22	27–48	Acterol (conc.) 1–10,000; 1 gtt.	0
4	II/11/30. Complete	31	27–45	Acterol (conc.) 1–10,000; 1 gtt. III/5–III/9	++ Healing +++
6	II/11/30. Complete	31	27–45	Acterol (conc.) 1–10,000; 1 gtt. III/5–III/9	+++ Healing ++++
7	II/11/30. Only one parathyroid in sections	22	25–41	None	++
8	II/11/30. Control	31	26–66	Acterol (conc.) 1 gtt. III/5–III/9	++++ Healing ++++
9	II/11/30. Control	31	26–62	Acterol (conc.) 1 gtt. III/5–III/9	++++ Healing ++++

Irradiated ergosterol (1 drop of a 1–10,000 dilution of Mead Johnson's concentrated Viosterol, stated to have an anti-rachitic potency 10,000 times that of cod liver oil) gave complete protection in 3 parathyroidectomized rats, and brought about healing after 5 doses in 2 rats.

It seems proved by these experiments that the antirachitic action of cod liver oil and of Viosterol is effective in the absence of the two principal parathyroid glands.

The possibility of accessory functional parathyroid tissue deserves consideration. Whereas Erdheim (9), in serial sections of the neck organs, found in every instance, minute collections of parathyroid cells, especially abundantly near the apex of the thymus or within its substance, Farner and Klinger (10), on the other hand, were able to demonstrate accessory glands in only 6 out of 35 serially sectioned animals, and in another series, in but 2 out of 64 rats. Bayer and Form (11) found them completely absent. In 6 of our experimental animals, complete serial sections were made of the neck organs, and in no instance could accessory parathyroid tissue be discovered. It would appear then that in the hooded strain used for these experiments accessory parathyroid tissue occurs exceptionally, if at all, and this possible source of error in the interpretation of the results may be definitely excluded.

In spite of the completeness of the removal of all parathyroid tissue, frank tetany did not develop in any of the animals, and the mortality, aside from deaths during the operation, has been low. Hammett (12) has shown that different strains of rats react differently as regards development of post-operative tetany and mortality. It is possible that the high calcium content of the Steenbock rickets-producing diet may have exerted a protective influence.

II. The Effect of Thymectomy

The literature bearing upon the relation of the thymus gland to the skeletal system is voluminous, but conflicting. In most of the older work indicating an influence of thymectomy upon the calcification of the bones, neither dietary nor hygienic factors were taken into consideration. Pappenheimer (13) was unable to confirm the alleged production of rickets in rats following thymus extirpation.

In spite of the lack of convincing evidence that the thymus has a definite relation to bone development or calcification, the recent report of Nitschke (14), that injection of thymus extracts produces a marked fall in blood calcium and inorganic phosphate, brings up the possibility that antirachitic agents may be modified in their effect in the absence of this tissue.

In the experiment shown in Table V, which is illustrative of several others, it is shown that removal of the thymus does not interfere with the protective or curative action of cod liver oil in experimental rickets.

Result of Experiment Shown in Table V.—Complete protection of thymectomized Rats 1, 5 and 8 with 2 gtt. daily of C.L.O., the control unoperated Rat 4 also being protected. Five doses of 2 gtt. of C.L.O.

TABLE V

Litter F

Steenbock Diet

Rat No.	Thymectomy	Days on diet	Weight	Treatment	Rickets
1	II/14/30. Complete	18	43–54	C.L.O. 2 gtt. daily	0
5	II/14/30. Complete	27	41–74	C.L.O. 2 gtt. II/20–III/12	0
8	II/13/30. Complete	27	44–71	C.L.O. 2 gtt. II/19–III/11	0
4	II/14/30. Control unoperated	19	41–42	C.L.O. 2 gtt. II/20–III/5	0
10	II/14/30. Complete	38	34–72	C.L.O. 2 gtt. III/13–III/17	+++ Healing ++
6	II/14/30. Control unoperated	38	41–107	C.L.O. 2 gtt. II/13–III/17	+++ Healing ++++
7	II/14/30. Control unoperated	38	32–64	C.L.O. 2 gtt. III/13–III/17	+++ Healing ++

brought about marked healing in thymectomized Rat 10, as well as in the unoperated controls, Rats 6 and 7.

III. The Effect of Combined Parathyroidectomy and Thymectomy

It seemed of interest to ascertain whether irradiated ergosterol would retain its antirachitic activity in the absence of both parathyroids and thymus. The experiment reported in Table VI shows that such is the case.

Serial sections were made of the excised parathyroids and of the entire neck tissues removed at autopsy. The completeness of the extirpation of both parathyroids and thymus was proven.

One may deduce from the experiment that Acterol is antirachitic in the absence of both thymus and parathyroids.

TABLE VI

Litter I

Steenbock Diet

Rat No.	Thymectomy	Parathyroid-ectomy	Days on diet	Weight	Treatment	Rickets
4	III/25/30	III/31/30	27	66–108	Acterol (1–10,000) 1 gtt. daily IV/1–IV/19	0
5	Control—unoperated	—	27	57–83	None	+++
6	Control—unoperated	—	27	71–98	None	+++

CONCLUSIONS

Cod liver oil and Viosterol in therapeutic doses are antirachitic in the absence of the parathyroid gland, or of the thymus, or of both.

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SPECIFIC POLYSACCHARIDES FROM FUNGI

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(Received for publication, July 16, 1930)

This report is concerned with the preparation and properties of a polysaccharide fraction from several yeast-like fungi and a trichophyton.

Since the recognition by Heidelberger and Avery (1) of the carbohydrate nature of the soluble specific substance derived from pneumococci, specifically precipitable polysaccharides have been isolated from a number of bacteria (2). Relatively little study has been made of the fungi, however. As long ago as 1894, Salkowski (3) prepared from brewers' yeast a gum which was subsequently described chemically (4). Using Salkowski's method as well as a modification thereof, Mueller and Tomcsik (5) obtained a gum from yeast which contained 0.19 per cent of nitrogen, was non-antigenic, but which gave a precipitin ring in a dilution of 1:800,000 with homologous rabbit antisera. Kurotchin and Chu (6), using a *Monilia tropicalis* isolated from a case of bronchomoniliasis, obtained complement fixation and weak precipitin tests with an alkaline extract of the monilia, and with the alcoholic precipitate from this extract. No further study of this material was reported.

The present progresive interest in medical mycology suggested the investigation of some of the fungi as to polysaccharide content. The following five yeast-like fungi and a trichophyton have been studied to date:

1. A monilia isolated from an interdigital erosion and identical culturally and morphologically with *Monilia psilosis*.

2. *Monilia psilosis*, Ashford, selected because of its possible relation to tropical sprue. The isolation of a carbohydrate from this organism has been briefly reported (7).

3. A monilia similar to *M. parapsilosis*, isolated from moist intertrigo of the toes.

4. A member of the genus *Willia*, obtained from an erosio interdigitalis.

5. A stock strain of *Saccharomyces cerevisiae*. (Commercial yeast was discarded as a source of material because of the presence of miscellaneous yeast-like fungi with the saccharomyces.)[1]

6. A strain of *Trichophyton gypseum* isolated from a trichophytosis of the hand.

Preparation of Polysaccharide Fraction

The organisms were cultivated on Sabouraud's honey broth until a heavy growth was obtained (1 to 3 weeks). With the exception of the trichophyton, the cultures were centrifuged, washed with water, followed by alcohol, and extracted overnight twice with ether. After drying, the organisms were ready for extraction. The trichophyton mats were washed with water, allowed to stand in alcohol overnight, and were then ground moist in a meat-grinder. The ground mass, after another washing with alcohol, was extracted overnight twice with ether, dried, and reduced to a fine powder in a ball mill.

The method of extraction was essentially that of Salkowski as given by Mueller and Tomcsik (5) in their work on yeast gum. It was, however, slightly modified in that the organisms (after alcohol washing and ether extraction) were given a preliminary treatment with Fehling's solution with the object of removing protein dissolved by the alkali. The same extraction process was found to be successful with all six organisms, with certain minor differences in the case of the trichophyton, (No. 6), as indicated in the following summary of the process.

10 gm. of organisms were suspended in water (50 to 100 cc.) and approximately 100 cc. of Fehling's solution were added. After standing 15 minutes the violet (No. 6—grey) precipitate was washed with water by centrifugation and extracted twice with 1:10 HCl. The thoroughly centrifuged acid solution (approximately 100 cc.) was partially neutralized with strong NaOH, and the gum precipitated with Fehling's solution (30 to 50 cc.). If after standing several minutes the gum did not come down, concentrated HCl was added drop by drop, but not to neutrality. At some point, probably dependent on ion concentration, the gummy precipitate formed. It was washed twice by pressing out in water (excessive water and handling were avoided as the precipitate easily became colloidal), and dissolved in 1:10 HCl (10 cc.). 95 per cent alcohol was added just to the point

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[1] We are indebted to Miss R. W. Benham of the Department of Dermatology, College of Physicians and Surgeons, Columbia University, for this information, and also for kindly furnishing the original cultures of the various organisms used.

of distinct permanent cloudiness and the precipitate removed by centrifuging. Except in the case of No. 6 this removed the opalescence, probably due to protein. Approximately three times the original volume of alcohol was then added, and the precipitate allowed to settle several hours followed by centrifugation. After washing in acidified alcohol the precipitate was redissolved in 1:10 HCl, again fractionally precipitated with alcohol, washed with alcohol and ether, and dried. The yield based on dry weight of the original material was about 1 per cent.

Properties of the Polysaccharide Fractions

The final products are in the form of a white (No. 6—pale tan) friable material or powder, which gelatinizes and slowly dissolves in water. They are precipitated by alcohol from dilute acid solution, and from aqueous solution by glacial acetic acid. As tabulated in Table I, the fraction obtained from each organism gives a Molisch test in a dilution of 1:1,000,000. The usual protein tests are, in general, negative. A very faint bluish-red biuret is obtained, however, in 1 per cent solution on standing several minutes, and the solid material from Organism 1 gives a positive Millon. All the preparations contain small amounts of nitrogen,[2] but the negative character of the protein tests would indicate that very little if any, of this nitrogen is present as protein. Sufficient material has not been available to do Van Slyke amino-nitrogen determinations.

The fractions are precipitated by Fehling's solution in the presence of sodium chloride as copper-containing compounds. When boiled with Fehling's solution they do not reduce it, but after 15 to 30 minutes hydrolysis with N/1 HCl at 100°C., reducing sugars are present. From each hydrolysate an osazone was prepared and identified as glucosazone by its crystalline structure. Although basic lead acetate yields with an aqueous solution of each fraction a white flocculent precipitate, insoluble in excess, no change in color is produced in a dilute iodine solution on addition of the polysaccharides, nor are they precipitated from aqueous solution by saturation with $(NH_4)_2SO_4$. Glycogen is accordingly absent. The difficulty of obtaining material in sufficient quantity has hindered more complete investigation of the chemical nature of the fractions, but from the above results it would

[2] Dumas micro-nitrogen determinations were kindly done in the laboratory of Prof. J. B. Niederl, Washington Square College, New York University.

TABLE I

Chemical Properties of Polysaccharides

Test used	Polysaccharide from organism					
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
1. Molisch on 1:1,000,000 solution	+	+	+	+	+	+
2. Robert's ring on 1 per cent solution	0	0	0	0	0	0
3. Trichloracetic acid saturated with $MgSO_4$, ring test on 1 per cent solution	0	0	0	0	0	0
4. Biuret on 1 per cent solution, after allowing to stand several minutes	±	±	±	±	±	±
5. Hopkins Cole on 1 per cent solution	0	0	0	0	±	0
6. Xanthoproteic on solid material	±	0	0	0	0	±
7. Millon on solid material	+	0	±	±	0	0
8. $(NH_4)_2SO_4$ to saturation of 5 per cent solution	0	0	0	0	0	0
9. Glacial acetic acid with 1 per cent solution	Ppt.	Ppt.	Ppt.	Ppt.	Ppt.	Faint ppt.
10. Basic lead acetate with 1 per cent solution	"	"	Faint ppt.	"	"	" "
Excess	"	"	" "	"	"	" "
11. Neutral lead acetate with 1 per cent solution	0	0	0	0	0	0
12. Dilute iodine solution	0	0	0	0	0	Blue green solution
13. Fehling's test	0	0	0	0	0	0
After acid hydrolysis, 100°C., 15 minutes	0	+	0	0	0	0
After acid hydrolysis, 100°C., 30 minutes	+	+	+	+	+	+
14. Phenylhydrazine reaction on hydrolysate	+	+	+	+	+	+
15. Nitrogen content, Dumas micro-method	1.93%	0.58%	0.53%	1.44%	1.60%	1.78%

± = Very faint or doubtful reaction.

appear that they are polysaccharides and non-protein, though the presence of the nitrogen makes it impossible to say this with finality.

Specific Precipitability of the Polysaccharides

That the isolated carbohydrates of bacteria are non-antigenic in the sense of inducing antibody formation has been abundantly demonstrated, particularly by Zinsser and Parker (8), by Mueller and Tomcsik (5) for the gum obtained from commercial yeast, and by Avery and Morgan (9) for the pneumococcus. In the absence of large quantities of these fungus polysaccharides no attempt has been made to test their antigenicity. It has been assumed in the light of previous work that they are non-antigenic. They are, however, with the exception of No. 6, precipitable by homologous antisera, and to a certain extent by antisera prepared against allied organisms. This is of interest in view of the specificity of most of the other polysaccharides heretofore isolated. The precipitability of the polysaccharides, even in high dilution, was apparently undiminished after heating 30 minutes at 100°C.

Antisera were prepared in rabbits by intravenous injections of suspensions of heat-killed organisms. From five to seven injections of 1 to 4 cc. of a 2 per cent suspension at 5 day intervals usually sufficed to produce an antiserum which would completely agglutinate the homologous organism in a serum dilution of 1:500. Agglutinin titrations were done in small test tubes against a fresh 1:100 or 1:200 suspension of a 24 hour Sabouraud's honey agar culture. The agglutinations were read twice, once after gentle rocking for ½ hour at room temperature and again after standing overnight in the icebox and a second similar rocking.

No attempt was made to agglutinate *Trichophyton gypseum* (No. 6) because its growth consists essentially of filaments, but the sera obtained after a series of injections of this organism were tested for precipitins against a saline extract of the organism. No precipitate was obtained, nor was the trichophyton polysaccharide precipitable with these or any of the other sera. It is accordingly omitted from consideration in the remainder of the paper.

The results of the agglutination tests are given in Table II. Each serum vigorously agglutinated the homologous organism, and in addition a number of cross-agglutinations were evident. Organism 2 was strongly agglutinated by Serum 1, and *vice versa*. This is in harmony with the cultural and morphological identity of the two. Strain 3 was also agglutinated by Serum 1 (subsequently sharply separated from Strains 1 and 2 by agglutinin absorption). Serum 3 strongly agglutinated Organisms 1, 3, and 5. Serum 5 also contained a

variety of agglutinins, reacting strongly with the *Willia* (No. 4) as well as with its own allied organism.

Using these sera, precipitin tests were carried out on serial dilutions of the several carbohydrates.

The sera were diluted 1:1 with saline before use. The polysaccharides were dissolved in saline, and the resulting solutions were overlaid on the sera. The

TABLE II

Agglutinin Titer of Unabsorbed Sera

Organism	Serum					Normal		
	1	2	3	4	5	A	B	C
1. *Monilia* from erosio	1:500*	1:500	1:200	0	1:50	1:10?	0	0
2. *M. psilosis*	1:200	1:500	1:50	0	1:10?	0	0	0
3. *M.* from intertrigo	1:500	1:10?	1:500	1:10	1:100	0	0	0
4. *Willia*	1:10	0	1:10	1:200	1:200	0	0	0
5. *Saccharomyces cer*	1:50	0	1:200	1:200	1:500	0	0	1:10?

* Highest serum dilution showing definite agglutination.

TABLE III

Precipitin Tests with Unabsorbed Sera

Organism	Serum					Normal		
	1	2	3	4	5	A	B	C
1	1:1,000,000*	1:100,000	1:10,000	0	1:10,000	0	0	0
2	1:1,000,000	1:1,000,000	1:10.000	0	1:10,000	0	0	0
3	1:100,000	0	1:1,000,000	0	1:100,000	0	0	0
4	0	0	0	1:1,000,000	1:100,000	0	0	0
5	0	0	1:10,000	1:1,000	1:1,000,000	0	0	0

* Highest dilution of polysaccharide giving precipitin ring.

presence or absence of precipitin rings was noted at the end of 5 minutes and 1 hour at room temperature, following which the two layers were mixed, the tubes placed in the icebox overnight, and a final reading was made the following morning after centrifuging if necessary.

The precipitin tests are recorded in Table III. Precipitin rings were regularly obtained between each serum and the homologous polysaccharide in dilution of 1:100,000 in 5 minutes, and in dilution of

1:1,000,000 in 1 hour. Cross-precipitins were found to roughly parallel the agglutinins with certain quantitative differences. In other words the polysaccharides isolated from these five yeast-like fungi would seem to lack the specificity observed in those from most bacteria heretofore investigated. By means of careful absorption, however, it was found possible to demonstrate a high degree of specificity. Whole serum was used for absorption together with strong suspensions of the organisms, and the procedure was similar to that used in testing for agglutinins. The dose of organisms varied with the quantity of antibody present. Just sufficient (usually 0.125 to 0.25 cc. of a 1:1 suspension per 1 cc. of serum) was used to completely absorb the precipitin for the absorbing organism's poly-

TABLE IV

Precipitin Absorption

Organism	Serum				
	1	2	3	4	5
1	Complete	Partial	0	—	0
2	Partial	Complete	0	—	0
3	0	—	Complete	—	0
4	—	—	—	Complete	0
5	—	—	0	0	Complete

saccharide, following which the centrifuged serum was re-tested for precipitin content against the other polysaccharides.

In Table IV there is indicated the ability of the several organisms to absorb the precipitin of a given serum for its homologous polysaccharide. It will be noted that in Sera 3, 4, and 5 only the homologous organism of each had this ability. Furthermore, a cipher indicates that although the precipitin for the polysaccharide of that particular heterologous organism had been completely absorbed, no absorption of the homologous precipitin could be detected. Accordingly the polysaccharides from Fungi 3, 4, and 5 are actually specific, each for its own organism. They are also quite different from Polysaccharides 1 and 2. Between these two, however, there is a somewhat less marked difference. Organism 2 was able to partially absorb the

precipitin for Polysaccharide 1 in Serum 1, and Organism 1 partially absorbed the precipitin for Polysaccharide 2 in Serum 2. This is especially significant in view of the cultural and morphological identity of the two organisms. Investigation of the two absorbed sera for evidence of absorption of agglutinins (Table V) was less significant. Organism 2 had only partly absorbed the agglutinin from Serum 1 for Organism 1, but the converse was not true, inasmuch as Strains 1 and 2 were found to be equally capable of absorbing all the agglutinin for Strain 2 from Serum 2. That this difference in the polysaccharides of Strains 1 and 2 signifies an actual difference, fundamentally speaking, in the two organisms, is doubtful in view of

TABLE V

Agglutinin Absorption

Organism	Serum				
	1	2	3	4	5
1	Complete	Complete	0	—	0
2	Partial	"	0	—	0
3	0	—	Partial	—	0
4	—	—	—	Partial	Partial
5	—	—	0	"	Almost complete

the identity of the two in other respects. One is inclined to discount also the difference in nitrogen content of Polysaccharides 1 and 2 (No. 1—1.93 per cent; No. 2—0.58 per cent), as well as the positive Millon reaction of Polysaccharide 1, as due to variation in impurities. It is planned, however, to make a further study of several additional such monilias to ascertain if similar distinctions can be consistently demonstrated.

Organism 1 and another stock strain of *Monilia psilosis* have been investigated, together with several allied strains, by Hopkins and Benham (10). They too observed cross-agglutination, but were able to distinguish between the two by agglutinin absorption. Subsequent work to be reported (11) inclines them to recognize a group of these organisms, culturally and morphologically similar, but exhibiting inconstant differences when studied by absorption of agglutinins.

Sera 3, 4, and 5, after suitable absorption of precipitins, were also investigated for agglutinating power, and in general, absorption of agglutinin paralleled absorption of precipitin. In Sera 4 and 5, however, a somewhat similar relation held to that in Sera 1 and 2. The absorption of precipitins was sharply limited to the homologous organisms as mentioned previously, but Organism 5 partially absorbed the agglutinin for Strain 4 from Serum 4, and Strain 4 was found to have partly absorbed the agglutinin for Strain 5 from Serum 5.

Certain quantitative differences were also noted between the absorption of precipitins and agglutinins. As already stated absorption was so adjusted that all the detectable precipitin for the polysaccharide of the absorbing organism was removed. The corresponding agglutinin, however, frequently had not been completely removed. Also unabsorbed Serum 1 was able to partially agglutinate Strain 5, but no precipitin for the corresponding polysaccharide could be demonstrated. Such differences, however, do not necessarily indicate the presence of two distinct antibodies. A difference in the quantitative relationships between antibody and antigen requisite for the demonstration of the two phenomena would explain, without assuming the presence of two antibodies, such discrepancies as are not due to variability inherent in the methods. Less antibody would seem to be necessary for the production of the antigen-antibody complex when the whole organisms serve as antigen than when the polysaccharide has this function. However, the polysaccharide-antibody complex would appear to be the more specific. As additional material becomes available it is hoped that more definite evidence on this question will be obtained.

SUMMARY

From each of five yeast-like fungi and a trichophyton there has been prepared a fraction which appears to be essentially a polysaccharide.

Tested by direct precipitation against the corresponding antisera the polysaccharides from the yeast-like fungi exhibit only partial specificity. Cross-precipitin reactions are frequent. By absorption of precipitin on the intact mycotic bodies, however, a relatively high degree of specific precipitability can be demonstrated for the polysaccharides.

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STUDIES ON MOUSE LEUKEMIA*

III. A Comparison of Four Lines of Leukemia Transmitted by Inoculation

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(Received for publication, July 21, 1930)

In previous communications we have recorded the existence of an inbred strain of mice designated as C58 in which lymphatic leukemia occurs spontaneously with great frequency, and also the transmission of leukemia to normal young mice of the same strain by inoculation with suspensions of minced tissues (1). We also reported the establishment of several lines of experimentally transmitted leukemia, by transfer from one mouse to another (2). These lines have been designated by letters of the alphabet in the order of their first transfers.

Elsewhere (3) we reported the results of inoculation of mice which were genetically different, *i.e.*, mice of the susceptible strain C58, a resistant strain (StoLi), the F-1 hybrids of these strains, and the back cross generations. Differences in the results were obtained which were interpreted as due to differences in the genetic constitutions of the hosts.

In this paper we report differences in results of inoculation due to differences not in the hosts, but in the materials inoculated.

With the establishment of several lines of experimentally transmitted leukemia, we found certain lesions appearing in particular lines with such frequency that they could be considered as characteristic of the lines. The differences between the lines were mainly those of ex-

* This investigation was supported by a grant from the Carnegie Corporation and an appropriation for technical assistance from the Research Fund of Columbia University.

The inoculation and supervision of animals were in the hands of Miss Mary Van S. McCoy and Miss Katherine Hill in New York.

tent or distribution of lesions, or the consistency of results. We have, for example, observed that typical leukemic blood counts were more common in certain lines than in others; that pleural or peritoneal effusions were, for a long time, confined to certain lines; that when inoculated into hybrids of the back cross generation, Line I showed fairly consistent proportions of susceptible mice, while the proportion susceptible to Line A varied when tested at different times; that the liver and kidneys presented differences in the frequency and degree of infiltration.

Four lines (A, E, H, I) have been carried for a sufficient number of transfers to furnish data on the relative frequency of the occurrence of some of these characteristics. We have selected for this purpose six features for comparison. These are the frequency of leukemic blood counts, pleural and peritoneal effusions, infiltration in liver and kidney, and the variability of results. The four lines were carried on simultaneously, although Line A was in the 10th transfer when the first transmission of Line I was made. The inoculations were made into mice taken at random from the closely inbred Strain C58, and animals of the same litter were frequently inoculated with material from different lines. From this genetic uniformity of the mice, it follows that differences between the lines can not be attributed to differences in the mice.

Leucocyte Counts

We have previously reported (2) that in spontaneous leukemia the leucocyte count is not always increased, and when increased does not always remain so. Similar variations have also been observed in leukemias transmitted by inoculation. The presentation of data on the occurrence of increased leucocyte counts therefore involves certain difficulties, for it has not been possible to examine the blood of each mouse at short intervals.

Daily determinations of the leucocyte counts in Line I have shown that significant changes do not occur until within 3 days of death, at which time sudden increases are usually observed. In other lines, marked changes do not occur within this interval. It is obvious, therefore, that to determine the frequency of this change, the counts must be made within this 3 day period. In many instances the blood

of only one mouse of a group inoculated with the same material was examined within this time, and this was usually the animal used as donor for the next transfer. Donors are killed instead of waiting for death to occur spontaneously, and this is usually done a day before the death of other mice in the same experiment. Terminal drops in the count are avoided by using only the highest count observed for each mouse within 3 days of death. The first eight transfers of each line are omitted because during this period the counts of Line A varied

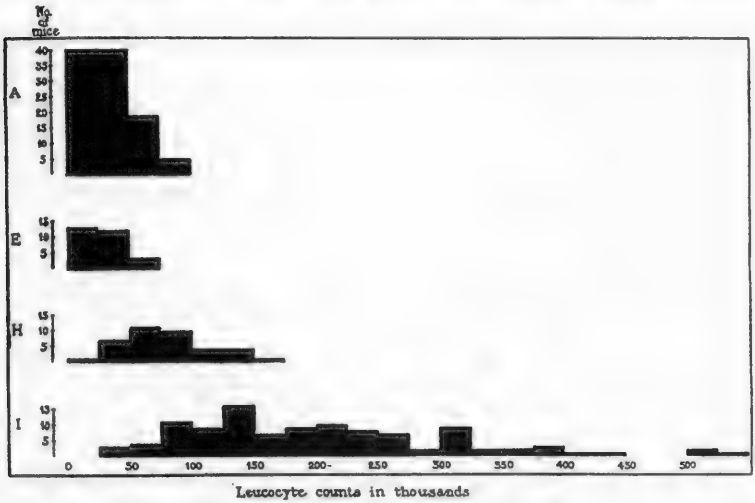

FIG. 1. Leucocyte counts in 4 lines of inoculated leukemia. All counts made within 3 days of death. Counts of the donors of these lines were: for Line A, 694,000 per cubic millimeter; Line E, 90,000; Line H, 292,000; Line I, 42,000.

considerably, and were higher than those which later became characteristic of the line. Limitation of data in this way does not affect the results except in Line A.

A comparison of the leucocyte counts thus tabulated (Fig. 1), shows that increases were observed less frequently in Lines A and E than in Lines H and I, and that the increased counts in the former lines were relatively less marked than in the latter. In Line I the counts were invariably increased and were frequently higher than any counts ob-

served in the other lines. The blood counts after inoculation cannot be correlated with the number of leucocytes in the blood of the spontaneous case used as donor for the first transfer of the line.

Peritoneal Effusion

In the early transfers of Line A several mice were found to have fluid in the peritoneal cavity. In most instances this was a milky fluid tinged with blood, and was often abundant. The number of mice that had this lesion increased so that after the eighth transfer, nearly every mouse dying of leukemia had peritoneal effusion. Specifically, from the 11th to the 23rd transfers inclusive, fluid was present in every positive case. In the 28th and subsequent transfers a smaller number of cases with fluid were observed, and the amount was less. Since the 28th transfer the appearance of the lesion has been inconstant and the amount relatively small. The great majority (83 per cent) of the cases in the first 43 transfers had fluid in the peritoneal cavity (Table 1).

In Line E the fluid first appeared in the third transfer, and reappeared in every subsequent transfer (but not in every mouse) through the 14th. During the next 10 transfers (40 mice) only five cases with fluid were found. The lesion was present in 27.6 per cent of the cases in the first 24 transfers.

In Lines H and I peritoneal effusion has been observed very rarely.

There are thus marked differences in the four lines with respect to the occurrence of this lesion. In Line A it was very frequent, in Line E less so, and in Lines H and I relatively rare.

None of the four spontaneous cases used as donors for these lines had fluid in the peritoneal cavity.

Pleural Effusion

As in the case of peritoneal fluid, that in the pleural cavity is milky, but usually with less admixture of blood. This feature has been observed frequently in Lines A (24.7 per cent) and E (35 per cent), but rarely in Lines H (0.7 per cent) and I (1.2 per cent) (Table 1).

The occurrence of fluid in the pleural cavity is less constant than in the peritoneal. It is very rarely observed in all the mice of an experi-

ment, and has varied at different times. In line A only 5 cases were observed in the first 17 transfers (4 per cent), and 80 cases in the next 24 transfers (38.7 per cent). In Line E there were 41 cases with pleural fluid in the first 14 transfers (43.6 per cent), but only 6 cases in the next 10 (15 per cent). The one case with pleural fluid that occurred in Line H was in the 10th transfer, and peritoneal fluid was present in the same case.

Pleural effusion was not present in any of the spontaneous cases used as donors.

TABLE 1

Occurrence of Peritoneal and Pleural Effusions in Four Lines of Inoculated Leukemia

Line	No. mice	No. transfers	Peritoneal effusion		Pleural effusion	
			No. mice	Per cent	No. mice	Per cent
A	343	43	285	83.0	85	24.7
E	134	24	37	27.6	47	35.0
H	144	26	4	2.8	1	0.7
I	504	37	3	0.6	6	1.2

Infiltration in the Liver

Infiltration in the liver varies in extent and position, and an arbitrary method of classification is necessary. In some animals the infiltration is extensive and present to the same degree in all microscopic sections. In such cases it is usually perivascular, with or without extensive intracapillary collections. These cases are classified as having "marked" infiltration.

Less marked degrees of infiltration are of different types. In some, the infiltration is widespread, but consists of fewer cells. In others it is not uniform, but may appear as solitary or multiple lymphomata resembling nodular metastases (sometimes seen in the gross specimen) or, more frequently, as perivascular collections appearing particularly around the portal vein near the hilum of the liver. The rest of the liver may be uninvolved, so that sections which do not include the porta hepatis with the larger vessels may not include the areas of infiltration. These several types of involvement are classified as "slight" infiltration.

Although examination of a single section does not exclude infiltration if none is found, it has been more convenient to examine but one section in most instances. The results of such examination show such differences in the four lines that conclusions can be drawn regarding the frequency of marked and slight degrees of infiltration.

Reference to Fig. 2 shows that in Lines H and I all of the livers examined were markedly infiltrated, as contrasted to Lines A and E in which some were markedly infiltrated, some had slight degrees of infiltration, and others had none in the section examined.

The livers of all of the four spontaneous donors had marked infiltration, a common finding in spontaneous cases.

Infiltration in the Kidney

Degrees of infiltration in the kidney are classified in the same way as in the liver. Line I had marked infiltration with great regularity, but no instances of slight infiltration. Line H had some cases with marked, but more with slight infiltration. In Lines A and E an extremely small number of cases presented infiltration in the kidney, although the perirenal fat was often densely infiltrated.

In spontaneous cases the kidney is infiltrated less frequently than the liver. Of the four spontaneous cases used as donors for these lines, infiltration in the kidney was found only in the donor for Line E, and this was of slight grade.

Variability

The consistency with which characteristic lesions have appeared has varied greatly in the different lines. Some of these variations have involved changes in the characteristic features of the lines, while others consist merely of greater or less uniformity among mice inoculated with the same material. Some of these changes have already been noted: in Line A, disappearance of marked increases in the leucocyte counts after the 8th transfer; diminution in number of cases with peritoneal effusion after the 28th transfer; variation in proportion of susceptible to non-susceptible back cross animals (3). In Line E, diminution in number of cases with peritoneal or pleural effusion after the 14th transfer. Corresponding changes have not been noted in Lines H or I.

Variation among mice in the same experiment has also differed in the four lines. In Lines H and I a considerable degree of regularity in result has been observed. Not only have the mice presented the same lesions, but the survival time, after the first few transfers, has varied only within narrow limits. In Lines A and E on the other hand, greater variation has been noted among mice inoculated with the same material, with respect to the occurrence of particular lesions and to the period of survival.

The differences between the survival times in Lines A and I are striking (Fig. 3). The transfers in Line I from the 16th to the 50th,

TABLE 2

Line	Marked infiltration	Slight infiltration	No infiltration
Frequency of infiltration of liver			
A	36	64	71
E	30	32	11
H	85	0	0
I	98	0	0
Frequency of infiltration of kidney			
A	0	1	98
E	1	1	68
H	20	36	28
I	87	0	10

The proportion of mice with various degrees of infiltration is indicated graphically in Fig. 2.

inclusive, involve 150 mice inoculated intraperitoneally, and cover the period from November 20, 1929, to June 8, 1930. During this period the survival time varied but little. With one exception, the mice died between 6 and 9 days after inoculation, the greatest number (47.3 per cent) on the 7th day. The 16th to 50th transfers of Line A involved 182 mice inoculated intraperitoneally. The range in survival time was from 6 to 71 days. The majority (85.2 per cent) died from 6 to 22 days after inoculation, but there was no great preponderance of deaths on any one day. If, instead of comparing the lines during the same

transfers, the comparison is made during the same time interval, the general shape of the frequency curve in Line A is unaltered and the marked difference in regularity persists.

Variability is also noted in the distribution of negative results in the four lines. In Lines E, H, and I, unsuccessful inoculations were

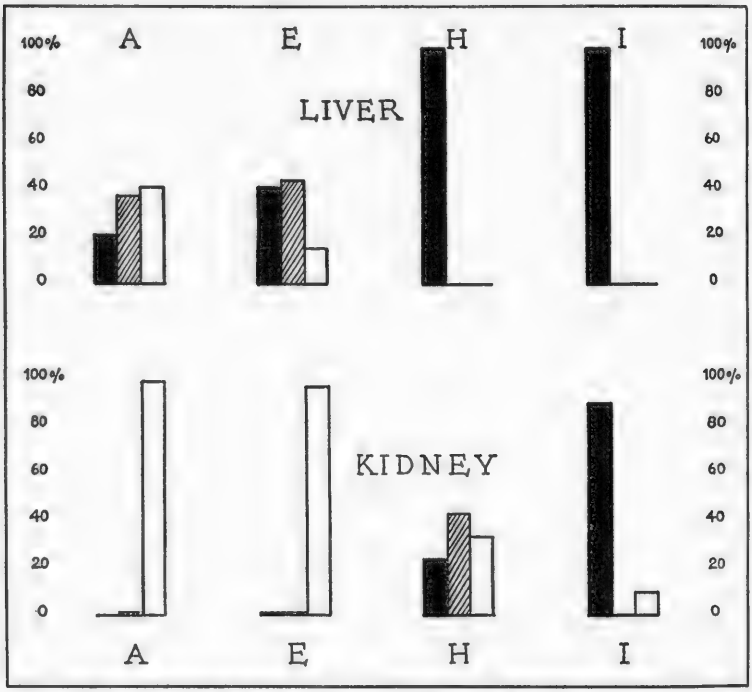

FIG. 2. Infiltration in liver and kidney. Degree of infiltration indicated as follows: black = marked infiltration; shaded = slight infiltration; white = no infiltration. Proportion of mice involved indicated by height of rectangles. Data for this table are given in Table 2.

obtained only in the first 3 transfers. In Line A, however, negative results were obtained in several later transfers, and as recently as the 35th.

Furthermore, Line A is the only one in which recoveries have ap-

parently occurred. During the period in which peritoneal effusion was an outstanding feature of the line, 5 mice that had swelling of the abdomen later recovered. Inasmuch as this abdominal swelling occurred only in the mice inoculated with Line A, and at the same time as in mice that later died with peritoneal effusion, it is believed that the temporary swelling was due to this lesion and that the mice recovered.

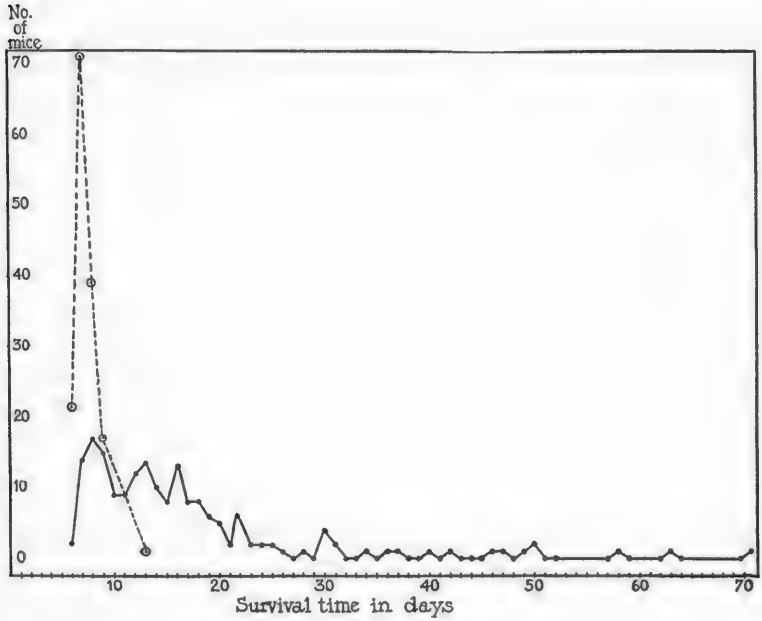

FIG. 3. Frequency curves of survival times of mice in Lines A and I.
Solid curve = Line A.
Broken curve = Line I.

DISCUSSION

The occurrence within a single inbred strain of mice of cases of leukemia presenting differences in the type, extent or distribution of lesions, and the appearance of the same differences within a single line of experimental transfers indicate that the fundamental process underlying the leukemic lesions may lead to varied manifestations.

In previous experiments (3) it was found that hybrid mice from the back cross to the non-susceptible strain were not all susceptible, but that among those which were, the type of response depended on the material inoculated and was the same in the hybrids as in mice of Strain C58 inoculated with the same material. That is, susceptible hybrids inoculated with material from Line A presented lesions found in C58 mice inoculated with Line A; those inoculated with Line I had lesions found in C58 mice inoculated with Line I.

It is evident that one of the important factors controlling the susceptibility to leukemia transmitted by inoculation is the genetic constitution of the host; yet the particular manifestations presented by susceptible animals depend not on their genetic constitution, but on the nature of the material inoculated.

SUMMARY

Several lines of lymphatic leukemia in mice, experimentally transmitted by inoculation into hosts of a closely inbred strain, have been established and carried on simultaneously. Among the inoculated mice there were found different types of response, according to the line of leukemia inoculated. The differences consisted mainly in the extent or distribution of lesions. Although the same line did not always show the same distribution of lesions, there was a distinct tendency for the cases in a line to present the same characteristics on successive transfers over a considerable period.

A comparison of the frequency of the occurrence of certain lesions in four lines of transmission shows that:

1. Increases in the total leucocyte counts were less common and less marked in Lines A and E than in Lines H and I. In the lines first mentioned, normal counts were relatively frequent. In Line H normal counts were uncommon, and in Line I were not observed. The highest counts were found in Line I.

2. Peritoneal effusion was characteristic of Line A for 43 transfers. It was found in Line E, but for a considerably shorter period and in a smaller proportion of cases; it was rare in Lines H and I.

3. Pleural effusion was frequently found in Lines A and E but rarely in Lines H and I.

4. Infiltration in the liver occurred to a marked degree in all the

mice of Lines H and I, but in fewer mice and to a variable degree in Lines A and E.

5. Infiltration in the kidney in Line I was present in 90 per cent of the mice, and was of marked grade. In line H it was present in 67 per cent of the mice (24 per cent of marked grade) but in Lines A and E it was rare.

6. A considerable amount of variability in results occurred in Lines A and E, whereas Lines H and I remained remarkably constant.

The lesions characteristic of a line were not necessarily those present in the spontaneous case from which the first transfer of the line was made.

As the mice used for inoculation were genetically uniform, the differences between the lines are not due to genetic differences in the hosts, but to differences in the materials inoculated.

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OBSERVATIONS ON NORMAL SYNOVIAL FLUID OF CATTLE

I. The Cellular Constituents and Nitrogen Content*

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Plates 37 and 38

(Received for publication, July 14, 1930)

A review of the literature concerned with the anatomy, histology, and physiology of normal joints reveals that our knowledge and understanding of many details is very meagre, indefinite, and inadequate. Therefore, we have undertaken studies which it is hoped will lead to a better understanding of the physiology of articulations. Further knowledge of the anatomy, histology and physiology of normal joints should have an important bearing on the physiology of pathological joints and the treatment of arthritis. Much of our work will concern the normal and pathological joints of cows, because of our ready access to an unlimited amount of material. This paper is the first of a series of studies of joints of cattle, and is a cytological study of normal synovial fluid.

A clear, viscid liquid known as synovial fluid is found in all normal joints. In the smaller laboratory animals, as well as in man, this fluid is present in such minute amounts that the aspiration of quantities sufficient for careful cellular and chemical study is difficult or impossible. The astragalotibial (hock) joint of normal cattle is a source of a large quantity of easily obtainable synovial fluid. Smaller amounts can be aspirated from the carpometacarpal (front knee) joint.

* The intensive study of arthritis at the Harvard Medical School has been made possible by friends and patients of the late Prof. Robert W. Lovett, who are giving a fund in his memory "to be devoted to the study of the most crippling disease."

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The following observations represent a part of the data obtained in a study of normal bovine synovial fluid.

Material and Methods

The synovial fluid was obtained by direct puncture of the astragalotibial and carpometacarpal joints of slaughtered animals, within 30 minutes after death. A lumbar puncture needle, with stylet in place, was inserted into the joint space through the lateral pouch of the capsule and synovial membrane. After the stylet was withdrawn, the fluid was aspirated into a Luer syringe. Most specimens were obtained after the legs had been skinned, although 10 of the astragalotibial joint aspirations were made through the intact hide from 1 to 8 minutes after the animals had been killed. In these 10 instances a special needle sleeve was used to puncture the tough hide overlying the joint. Since no apparent changes in the cellular constituents or nitrogen content of the fluids occurred in the 15 to 30 minutes after death, this procedure was abandoned.

Such specimens of synovial fluid were placed in warm test tubes, which were tightly corked and transferred to an inside shirt pocket at a temperature of approximately 33°C. On reaching the laboratory they were kept at a temperature of 38°C. In this manner the cells were kept viable for several hours.

Total cell counts per cubic millimeter of synovial fluid were made on 126 specimens. In order to distinguish more clearly the red blood corpuscles from the nucleated cells, a film of cresyl blue (dissolved in alcohol) was allowed to dry on the cover slip of the hemocytometer. When the synovial fluid flowed into the counting chamber this dye was dissolved in sufficient concentration to cause nuclear staining.

Differential cell counts were done on 40 synovial fluids by means of the supravital technique described by Sabin (1). The glass slides that were used were allowed to stand for 3 or 4 days in concentrated sulfuric acid to which a few crystals of potassium bichromate had been added. They were afterwards washed in running water, rinsed in three changes of distilled water, allowed to stand over night in distilled water, and then stored in 80 per cent alcohol. When taken from the alcohol, they were dried, flamed, polished with silk, and flooded with a stain which was prepared as follows: 30 drops of a saturated solution of neutral red in absolute alcohol and 7 drops of a saturated solution of janus green B in absolute alcohol were added to 10 cc. of absolute alcohol. This concentration of dye was found by trial to be the most satisfactory for our purposes. The clean glass slides were flooded with this freshly prepared stain and immediately placed in the vertical position to dry. For study, a single large drop of synovial fluid was placed on a prepared slide, covered with a clean rectangular cover-slip and sealed with a vaselin-paraffin mixture. Microscopic observations and cell counts were made in a warm box at 38°C. In addition many preparations were studied which were stained either with neutral red or with janus green B.

The phagocytic action of the various types of nucleated cells was observed in

12 synovial fluids. In these instances a graphite suspension was prepared in the manner described by Drinker and Churchill (2) and diluted with 0.85 per cent sodium chloride solution to obtain the desired concentration. This suspension was found to be superior to India ink because the particles are smaller and do not agglutinate. A few drops of this suspension were added to the synovial fluid either at the time of its withdrawal from the joint or when the fluid was placed on a similarly prepared glass slide. Observations were made as in other supra-vital preparations.

Total nitrogens on 150 fluids were done by the macro Kjeldahl method (3). The non-protein nitrogen was determined by the Folin method for blood (4) on 20 fluids. Total nitrogen determinations, before and after precipitation of mucin* (5), were made in 17 instances.

Presentation of Data

Because of constant differences in total cell counts, total nitrogen values, and physical properties of synovial fluid obtained from carpometacarpal and astragalotibial joints, the two types of fluids have been studied separately and tabulated. These variations, together with differences attributed to the age and habitat of the cattle, will be discussed in detail below. Tables I, II, and III represent cell counts and determinations made on synovial fluid from young western beef cattle.**

The average cell count on 63 astragalotibial joint fluids was 112 nucleated cells and 64 red blood corpuscles per cubic millimeter. A series of 15 carpometacarpal joint fluids from some of the same animals yielded an average of 222 nucleated cells and 572 red blood corpuscles per cubic millimeter. When 13 of the 78 specimens in which the red blood corpuscle count exceeded 200 per cubic millimeter were

* By mucin we mean the ropey, stringy substance precipitated by the addition of acetic acid to synovial fluid. The procedure used was: The synovial fluid was diluted with four parts of distilled water. For each cubic centimeter of undiluted synovial fluid 0.1 3 cc. of 7 normal acetic acid was added and the solution vigorously shaken.

** By beef cattle we mean young steers and heifers approximately 2 to 4 years of age. These animals were largely Herefords, shipped mainly from the western states. The milch cows referred to below were much older (estimated between 8 and 12 years of age) and obtained largely from the New England district. Since these studies were made during the winter months, it seems safe to consider milch cows as stable cattle and beef animals as pasture cattle.

discarded, the average cell count on the remaining 56 astragalotibial joint fluids became 103 nucleated cells and 29 red blood corpuscles per cubic millimeter. The remaining 9 carpometacarpal joint fluids then gave an average of 165 nucleated cells and 59 red blood corpuscles per cubic millimeter of fluid.

The average total nitrogen of 56 astragalotibial joint fluids was found to be 161 mg. per 100 cc. Total nitrogen determinations on 18 carpometacarpal joint fluids from the same group of animals yielded an average of 270 mg. per 100 cc.

In a separate series of 17 astragalotibial joint fluids an average total nitrogen value of 169 mg. per 100 cc. was found. After precipitation

TABLE I

A Summary of the Cell Counts Made on Seventy-Eight Fluids Showing the Maximum, Minimum, and Average Numbers of Erythrocytes and Nucleated Cells

Synovial fluids from astragalotibial joints							Synovial fluids from carpometacarpal joints						
Number of fluids	Erythrocytes per cmm.			Nucleated cells per cmm.			Number of fluids	Erythrocytes per cmm.			Nucleated cells per cmm.		
	Max.	Min.	Aver.	Max.	Min.	Aver.		Max.	Min.	Aver.	Max.	Min.	Aver.
63	510	0	64	252	55	112	15	3560	0	572	492	95	222
After discarding the specimens in which the erythrocytes exceeded 200 per cubic millimeter of fluid													
56	195	0	29	252	55	103	9	125	0	59	250	95	165

of mucin an average total nitrogen value of 132 mg. per 100 cc. was obtained. Non-protein nitrogen determinations on 14 fluids gave an average of 23.9 mg. per 100 cc. The above averages indicate that 37 mg. of the total nitrogen of synovial fluid is taken out by this method of mucin precipitation. When the 37 mg. thus accounted for and the 23.9 mg. of non-protein nitrogen were deducted from the average total nitrogen (169 mg. per 100 cc.), 108.1 mg. of nitrogen per 100 cc. remained. Multiplying 108.1 by the factor 6.25, one obtains 680 mg. or 0.68 per cent, which we believe is the approximate total protein content for normal astragalotibial joint fluid of cattle.

The total nitrogen was found to be constantly higher in the car-

pometacarpal joint synoviae than in the astragalotibial joint fluids. The cell counts also were consistently higher. There was no correlation between the total nitrogen content and the number of red blood corpuscles or nucleated cells per cubic millimeter of individual fluids.

Comparison of Carpometacarpal and Astragalotibial Joints

Gross differences were noted immediately when fluids obtained from the carpometacarpal and astragalotibial joints of the same or different animals were examined. The quantity of synovia obtainable from the carpometacarpal joints was smaller than the amount that could be aspirated from the astragalotibial joints. In young beef cattle an average of 3 to 7 cc. of synovial fluid was obtained from the carpometacarpal joints, whereas 15 to 40 cc. of fluid could be aspirated from the astragalotibial joints. The fluids obtained from the carpometacarpal joints were viscid and occasionally clotted. Astragalotibial joint synovia was much less viscid, being about the consistency of 6 per cent gum acacia solution. Coagulation never occurred in any of the astragalotibial joint specimens. As shown above, the total nucleated cell counts, as well as the total nitrogen content, were constantly higher in the carpometacarpal joint fluids than in the fluids from the astragalotibial articulations.

A probable explanation of these differences was found when the two joints were examined macroscopically and microscopically. Inspection of the opened carpometacarpal joints revealed constant defects in the articular cartilage. These defects varied in size. They were always situated on the medial side of the metacarpal bone and the opposing articular surface of the adjacent carpal bone (see Fig. 1). In gross these erosions were irregularly shaped depressed areas in the articular cartilage, apparently going down to the subchondral bone. The margins were sharp and in some instances slightly undermined. The bases frequently appeared to be covered by fibrin. Histological study revealed a sharply defined and abrupt area of cartilage degeneration, in some places extending down to bony trabeculae (Fig. 2). In other places one or two rows of distorted cartilage cells remained. At the base of these defects small blood vessels and capillaries nearly reached the surface. In some of the sections a moderate infiltration of small round cells and mononuclear phagocytes was seen. Because

of these constant defects in practically all cattle 2 years of age or older. the synovial fluid from these joints was considered pathological. The increased viscosity and higher nitrogen values of these fluids, together with their tendency to clot, seemed best explained on the assumption that there was altered capillary permeability in the abnormal joints and a possibility of filtration of fibrin into the joint spaces.*

Because of the fact that the carcasses are hung by the tendo-Achilles, direct inspection of the astragalotibial joints was impossible. In beef cattle, 2 to 4 years of age, incision into such joint spaces and palpation of the articular cartilages of the joints from which the fluids were withdrawn did not reveal palpable defects in any instance. Direct inspection of 6 astragalotibial joints of older stable cattle, that had been condemned, revealed 4 joints showing areas of beginning marginal degeneration of the articular cartilages For these reasons it was assumed that the astragalotibial joint synovia of young beef cattle was normal, but that the synovial fluid of the carpometacarpal joints of animals over 2 years of age must be considered pathological.

The quantity of synovial fluid obtainable from the joints of stable cattle (milch cows) is strikingly low as compared with young beef cattle. In the former an average of 2 to 3 cc. of fluid could be aspirated from the carpometacarpal joints and only 5 to 10 cc. were obtainable from the astragalotibial joints. Von Holst (6) noted that synovial fluid was obtained from a single joint of slaughtered cows in quantities varying from almost none to 15 or 20 cc. He also confirmed Frerichs' (7) earlier observation that much more fluid could be obtained from the joints of young or stall fed cattle than from the joints of grazing animals. From a single ox 65 cc. of fluid were withdrawn from the astragalotibial joint.

Types of Cells

There was no striking difference in the type of cells found in the synovial fluids from young beef or stable cattle. More cellular fragments, solid particles and unidentified débris were found in the fluids from stable cattle than in those of young beef cattle.

* A more detailed study of these articular cartilage lesions and the age at which they appear is being made.

Table II gives in detail the numbers and types of cells seen in the carpometacarpal and astragalotibial joint fluids obtained from the same animals. The cells have been divided into groups according to the classifications of Sabin, Doan, and Cunningham (8), (9), (10), and Key (11), (12). We have found that the nucleated cells of cows' synovial fluid correspond quite accurately to the cell types described by Key (11) in his study of normal rabbits' synovial fluid. It has been our purpose, however, to classify the nucleated cells as to their phagocytic properties without reference to their origin. We have limited ourselves to brief descriptions of the major groups of cells present.

Ninety to ninety-five per cent of all nucleated cells present were phagocytic for particulate matter and, as seen in a number of instances, for cells and fragments of cells. Fourteen macrophages were found in which 1 to 6 recognizable phagocyted cells were present. Cells of the monocyte series formed 85.5 per cent of all the nucleated cells, clasmatocytes 4.1 per cent, while 3.3 per cent consisted of other types of phagocytic cells.

The monocytes were as a rule easily recognized, though they varied greatly in size and degree of stimulation. They were almost invariably spherical or oval in shape and contained eccentrically placed nuclei. The nuclei were usually kidney-shaped, although sometimes oval, and contained a considerable amount of chromatin. In almost all instances there was an accumulation of neutral red granules and phagocyted particles in the cytoplasm near the nuclei on the indented side and often rosettes were seen at this point. Mitochondria in the form of dark green or blue rods and dots could usually be seen best at the poles of the nuclei.

The clasmatocytes were larger than the monocytes. They were almost always oval in shape although sometimes irregular in outline. The nuclei of these cells were relatively smaller and appeared more glassy. The granules and vacuoles which were stained by neutral red varied more in size and were more widely distributed than in the monocytes. Mitochondria when seen were in the form of small dots and rods, well scattered. Frequently in the double stained preparation mitochondria could not be seen.

Both of the above types of cells were actively phagocytic for graphite particles (Cells 9 to 13, Fig. 3).

Polymorphonuclear leucocytes were actively ameboid and phagocytic for particles of graphite. There was a varying number of phagocytic cells without sufficiently definite morphology to be classified in any special group. These cells have been called unclassified phagocytic cells.

Small round cells with relatively large, round, or slightly indented nuclei were occasionally seen. The cytoplasm of these cells was scant in amount and clear.

TABLE II

*A Comparison of Cells and Total Nitrogen Values of Synovial Fluids Obtained from the Carpometacarpal and Astragalotibial Joints of the Same Animals**

		Phagocytic cells Percentage							Non-phagocytic cells Percentage			Totals				
Animal number	Joints	Monocytes	** Small monocytes	*** Phagocytic monocytes	**** Large stimulated phagocytic cells	Clasmatocytes	Phagocytes unclassified	Polymorphonuclear leucocytes	Lymphocytes	Synovial cells	Cells unclassified	Phagocytic cells	Non-phagocytic cells	Nucleated cells per cmm.	Erythrocytes seen in counting 100 nucleated cells	Total nitrogen in mg. per 100 cc.
1	Front	54	14	1	12	8	2	1	2	5	1	92	8	110	9	195
2	Front	58	21	0	8	2	3	0	2	4	2	92	8	170	83	252
3	Front	61	7	0	11	7	3	1	1	5	4	90	10	160	104	241
4	Front	59	12	1	9	7	4	1	2	5	1	92	8	225	4	300
5	Front	63	9	0	10	8	2	0	1	5	2	92	8	187	47	351
Max.		63	21	1	12	8	4	1	2	5	4	92	10	225	104	351
Min.		54	7	0	8	2	2	0	1	4	1	90	8	110	4	195
Aver.		59	10.6	0.4	9.8	6.4	2.8	0.6	1.6	4.8	2	91.6	8.4	170.4	49.4	267.2
1	Rear	55	18	0	10	5	1	0	4	3	4	89	11	70	18	141
2	Rear	67	10	1	8	3	2	0	1	5	3	91	9	95	22	196
3	Rear	64	9	0	6	3	5	1	6	3	3	88	12	125	1	158
4	Rear	74	7	0	8	3	1	0	1	4	2	93	7	110	89	177
5	Rear	69	11	0	9	4	2	2	2	0	1	97	3	80	2	179
Max.		74	18	1	10	5	5	2	6	5	4	97	12	125	89	196
Min.		55	7	0	6	3	1	0	1	0	1	88	3	70	1	141
Aver.		65.8	11	0.2	8.2	3.6	2.2	0.6	2.8	3	2.6	91.6	8.4	96	26.4	170

* All specimens were taken from normal young beef cattle within 30 minutes after death. Corresponding animal numbers indicate the pairs of fluids.

** Small phagocytic cells classified as monocytes because of nuclear characteristics and accumulation of neutral red granules in the hof of nucleus.

*** Monocytes which had ingested recognizable cells.

**** Large stimulated cells which were phagocytic and resembled monocytes more than clasmatocytes.

The mitochondria, although sometimes scattered, were usually seen in one small area. These cells have been classified as lymphocytes.

A few cells interpreted as being synovial cells were found in most specimens. These were non-motile, varied greatly in shape and size, and contained relatively small oval nuclei, which were usually clear. The cytoplasm was always finely vacuolated and contained numerous finely distributed dark granules interspersed with the vacuoles. Granules stained with neutral red were never observed in these cells. In most instances these cells appeared to be degenerating.

COMMENT

Relatively few studies of the cytology of normal synovial fluid have been made.

Key, by means of supravital staining, was able to show that the synovial fluids of normal rabbits contained an average of 58 per cent monocytes, 15 per cent clasmatocytes, 14 per cent indeterminate macrophages, 5 per cent leucocytes, 1 per cent primitive cells and 3 per cent synovial lining cells. In all instances 85 per cent or more of the cells were macrophages. He found from 175 to 225 living cells per cubic millimeter. He further believed that red blood corpuscles were normally present in slight excess of the nucleated cells. In 1926 Key (14) attributed to the macrophages and leucocytes the function of removal of waste and foreign matter from the joint cavity. Hammer (15) washed out supposedly normal joints with Mueller's fluid and examined the sediment from the washings. He described the following: (a) Large round or irregular, vacuolated cells with one to three nuclei, which he believed to be desquamated synovial cells; (b) small round cells with little protoplasm; (c) nuclei of degenerating encapsulated cells; (d) cell rests, free nuclei and small granular masses of protoplasm; (e) thin membranes staining with carmine and showing fine striations; (f) threads with a homogenous or striated structure which had an affinity for hematoxylin and were devoid of cells; (g) elastic fibers; (h) large tissue masses and broken off villi of various kinds; (i) fat droplets; (j) red blood corpuscles.

Our studies show that the astragalotibial joint fluid of young beef cattle contains an average of 103 to 112 nucleated cells per cubic millimeter (see Table I), whereas the carpometacarpal joint fluid contains an average of 165 to 222 cells per cubic millimeter. We do not believe, however, that the carpometacarpal joint fluid can be considered normal. Of all the nucleated cells present 90 to 95 per cent have been shown to be phagocytic for particulate matter. We cannot agree with Key (11 and 13) that red blood corpuscles are normally present in numbers slightly in excess of the total number of nucleated cells. We have had three fluids in which no red blood corpuscles were seen in

TABLE III

*Total Cell Counts and Cell Types Contained in Normal Synovial Fluid Obtained from the Astragalotibial Joints of Twenty-One Young Western Beef Cattle**

	Phagocytic cells Percentage							Non-phagocytic cells Percentage			Totals			
Animal number	Monocytes	** Small monocytes	*** Phagocytic monocytes	**** Large stimulated phagocytic cells	Clasmatocytes	Phagocytes unclassified	Polymorphonuclear leucocytes	Lymphocytes	Synovial cells	Cells unclassified	Phagocytic cells	Non-phagocytic cells	Nucleated cells per cmm.	Erythrocytes seen in counting 100 nucleated cells
1	69	11	0	9	4	2	2	2	0	1	97	3	80	2
2	74	7	0	8	3	1	0	1	4	2	93	7	110	89
3	78	8	1	2	5	1	0	1	2	2	95	5	85	24
4	68	14	0	8	4	1	0	0	1	4	95	5	96	21
5	82	4	0	6	3	2	1	0	0	2	98	2	92	8
6	88	7	0	1	1	0	2	0	0	1	99	1	102	34
7	60	11	1	15	2	7	2	0	2	0	98	2	100	65
8	78	9	1	6	0	3	0	0	2	1	97	3	140	37
9	67	10	1	8	3	2	0	1	5	3	91	9	95	22
10	64	9	0	6	3	5	1	6	3	3	88	12	125	1
11	55	18	0	10	5	1	0	4	3	4	89	11	70	18
12	64	10	0	6	5	1	3	4	4	3	89	11	95	16
13	62	15	0	9	1	1	2	4	4	2	90	10	65	2
14	59	8	2	11	7	2	3	1	3	4	92	8	55	6
15	69	6	1	9	4	1	0	0	7	3	90	10	90	6
16	74	5	0	8	8	3	0	0	2	0	98	2	80	4
17	64	4	2	9	11	4	0	0	6	0	94	6	72	25
18	62	17	0	11	0	5	3	0	0	2	98	2	102	292
19	55	10	0	19	3	3	1	2	3	4	91	9	86	120

20	57	17	1	15	3	3	1	1	2	0	97	3	80	4
21	65	6	1	21	3	2	0	1	1	0	98	2	60	7
Max.	88	17	2	21	11	7	3	6	7	4	99	12	140	292
Min.	55	4	0	1	0	0	0	0	0	0	88	1	55	1
Aver.	67.3	9.8	0.5	9.4	3.7	2.4	1	1.3	2.6	2.0	94.5	5.9	90	(*)20.6 38.3

* These fluids were obtained in from 5 to 30 minutes post mortem.

** Small phagocytic cells classified as monocytes because of nuclear characteristics and accumulation of neutral red granules in the hof of nucleus.

*** Monocytes which had ingested recognizable cells.

**** Large stimulated cells which were phagocytic and resembled monocytes more than clasmatocytes.

(*) By not including the two high counts (292 and 120) the erythrocyte average was 20.6. Including these abnormally high counts the average was 38.3 erythrocytes to every 100 nucleated cells seen.

the entire ruled field of the counting chamber. Many fluids contained only 1 to 5 erythrocytes per cubic millimeter and in only 15 out of 78 specimens counted did the number equal or exceed the total number of nucleated cells. From our observations we believe that the number of red blood corpuscles present are in direct proportion to the trauma to which the joint is subjected. Key (11) obtained fluid by passing a capillary glass pipette through the shoulder muscles and joint capsule of a rabbit's shoulder joint and then flexed the limb so that increased intra-articular pressure forced the fluid to rise into the tube. We know from perfusion experiments that the synovial membrane through which a needle or pipette must pass to enter the joint cavity is extremely vascular. The rupture of a single capillary of the synovial membrane would be enough to give the number of red blood corpuscles reported by Key, to say nothing of the blood that would probably enter the unprotected end of the glass pipette in its passage through the shoulder muscles.

In addition to the recognizable cells present in synovial fluid, we have occasionally noted fragments of cells, rare curved fibrils and oval bodies. Some of these bodies, because of concentric lamina, suggested degenerating cartilaginous fragments. In old stable or milch cows the amount of cellular detritis and unidentified solid material was much greater than in the young beef animals whose habitat is pasture-lands rather than barn stalls. Occasional bloody or yellow fluid and in a few instances turbid fluids were obtained. These were omitted from our studies.

SUMMARY

1. The astragalotibial (hock) joints of normal young beef cattle contain large and uniform quantities of synovial fluid which is easily accessible for study.

2. Nucleated cells found in such synovial fluid are similar in numbers and types to those described previously in normal rabbits' synovia.

3. The fact that 90 to 95 per cent of all nucleated cells present are actively phagocytic implies that the function of these cells is the removal of the products of wear and tear from the articular cartilages and synovial membranes.

4. Red blood corpuscles seem to be present in numbers directly proportional to the trauma to which the synovial membrane is subjected.

5. The astragalotibial joints of young beef cattle contain from 3 to 5 times more fluid than do the corresponding joints of stable cattle. More débris and unidentified solid material is found in the latter group of animals.

6. The total nitrogen content of this synovial fluid was found to be 169 mg. per 100 cc. The approximate total protein content was calculated to be 680 mg. or 0.68 per cent per 100 cc.

7. No correlation between the total protein level and the total cell counts of isolated specimens of synovial fluid is possible from these data.

8. Further studies of synovial fluid and of its chemical similarity to blood serum of the same animals are in progress.

9. Articular cartilage defects occurring in the articular cartilages of the carpometacarpal joints are described. (A more detailed study of these articular cartilage lesions, the age at which they appear, possible causative factors, etc., is now being made.)

We wish to thank the New England Dressed Meat and Wool Company for their cooperation and generosity, without which this work could not have been possible.

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EXPLANATION OF PLATES

PLATE 37

FIG. 1. A photograph of the opened carpometacarpal joint (about ¾ natural size), showing the opposing areas of medial articular cartilage degeneration. A cartilage laceration, indicated by an arrow, is the result of a knife cut. Specimen obtained from a young western beef steer (C-3).

FIG. 2. Photomicrograph (low power) showing the margin of an articular cartilage defect illustrated in Fig. 1. Note the total absence of the articular cartilage and that the base of the defect is formed by subchondral bone.

PLATE 38

FIG. 3. Camera lucida drawings (× 1800) illustrating the cells seen in synovial fluid of normal cattle. The drawings were made from supravital preparations in which both neutral red and janus green B were used as the vital stains. The black particles illustrated in Cells 9–13 represent the graphite particles which had been ingested by the cells before they were supra-vitally stained. Entirely clear portions of cells represent those areas which remained unstained, neutral red staining is indicated by various shades of grey, while mitochondria are best seen in Cells 2 and 8 as minute dark rods.

Cells 1 to 4 are monocytes; 5, a phagocytic monocyte with ingested dead cell; 6 and 7 are varying sized clasmatocytes; 8, a lymphocyte showing a small clump of mitochondria but no neutral red staining; 9, clasmatocyte with ingested graphite particles scattered throughout the cytoplasm between neutral red stained vacuoles; 10 to 13 varying sized monocytes showing ingested graphite. Note the accumulation of neutral red stained vacuoles and graphite particles in the hof of the nuclei; 14 to 17, desquamated synovial cells with unstained vacuoles. When graphite was added to the fluids these cells showed no phagocytic properties.

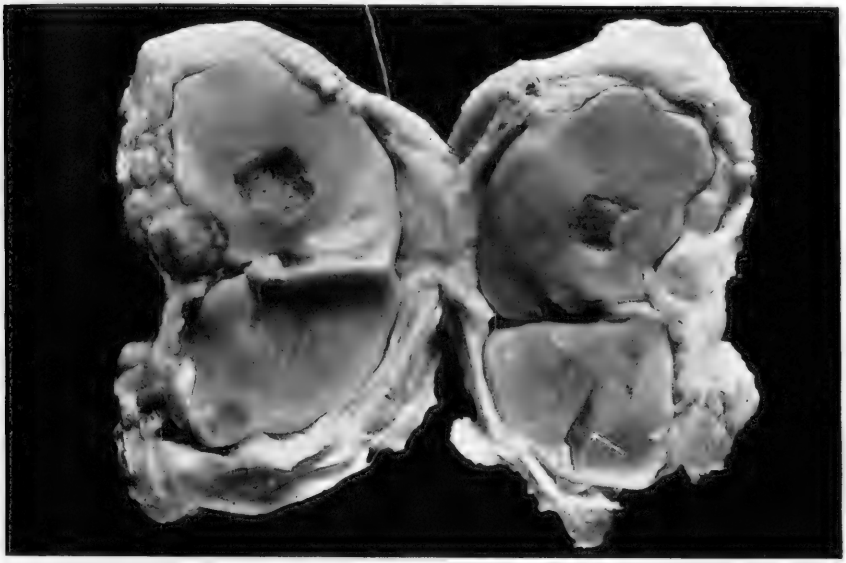

FIG. 1

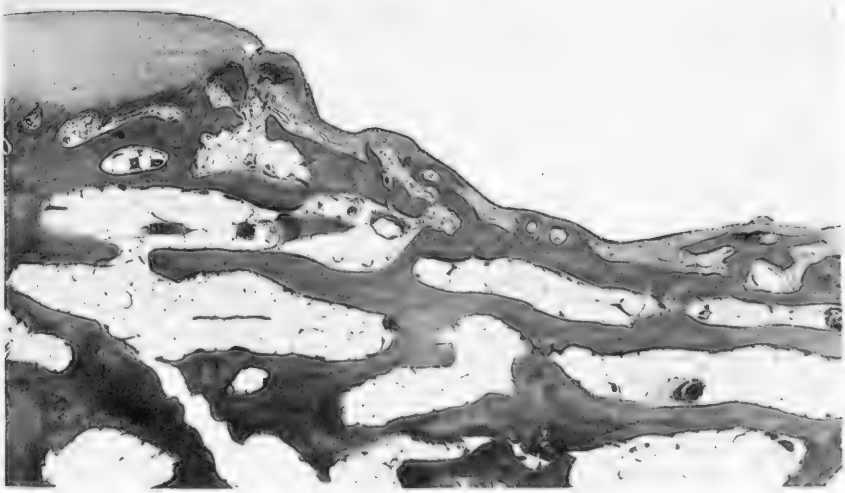

FIG. 2

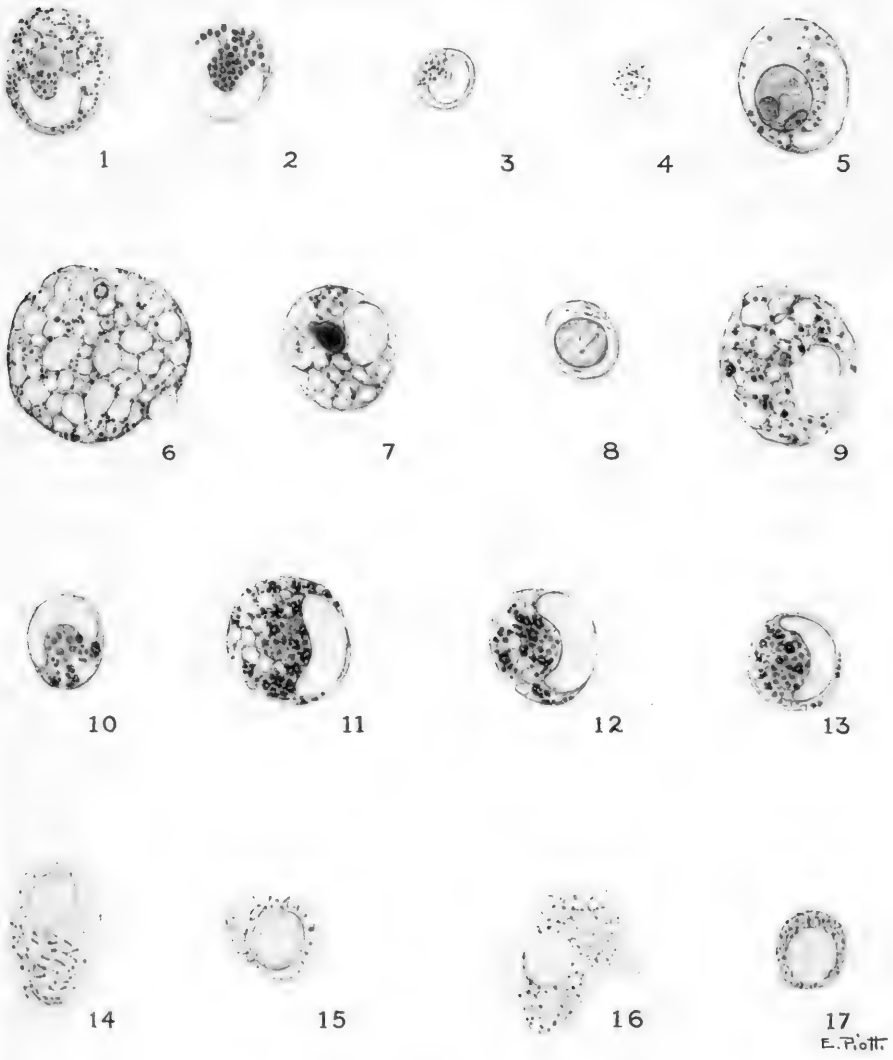

FIG. 3

THE PRODUCTION OF INFECTION WITH PNEUMOCOCCI IN RABBITS BY INTRADERMAL INOCULATION OF TYPE III PNEUMOCOCCI, THE TREATMENT WITH SPECIFIC ANTISERUM, AND A COMPARISON WITH TYPE I INFECTIONS

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(Received for publication, August 8, 1930)

The very favorable results from treatment of Type I pneumonias and the encouraging although less favorable results in Type II led the clinicians who were aiding in the general pneumonia investigation* to try serum treatment in Type III infections. As the observations and statistics of Bullowa,[1] Rosenbluth,[1] Park, Bullowa and Rosenbluth,[2] Cecil and Sutliff[3] of the treatment of patients with Type III pneumonia with specific antiserum did not indicate very favorable results, it seemed to us that it would be interesting to study the effect of the Type III antiserum in the treatment of pneumonia in experimental animals. The progress of the disease could be compared with that of Type I pneumonia in which serum treatment of patients has proved to be of value. In case the animals could be infected and cured, the relative amount of serum necessary could be found and might furnish data on which to base a plan of dosage for further clinical trial of the antiserum.

The production of an infection by intradermal inoculation and the subsequent intravenous inoculation of a single dose of antiserum was carried out by a technique slightly modified from that used by Good-

* This investigation was carried out under the direction of Dr. Park and was aided by grants of money from the New York University Littauer Pneumonia Fund and the Metropolitan Life Insurance Influenza Commission for part of the expense.

[1] *Jour. Am. Med. Assn.*, 1928, **90**, 1354, 1351.

[2] *Jour. Am. Med. Assn.*, 1928, **91**, 1503

[3] *Jour. Am. Med. Assn.*, 1928, **91**, 2035.

ner[4] in his Type I intradermal pneumonia experiments. Goodner showed that by the injection of a small amount of Type I pneumococcus culture intradermally a local lesion and a bacteremia could be produced and that the infection was fatal for about 96 per cent of the rabbits inoculated. He showed that the bacteremia and the development of the lesion could be checked and that recovery took place when suitable amounts of Type I antiserum were given. He suggested that this method be used for the standardization of Type I antipneumococcus serum.

For our experimental work in treating the Type III infections a concentrated Type III antiserum was used which was estimated by mouse protection tests to have 1000 units per cubic centimeter against the stock Type III strain. This strain was the one used for immunization of the horse from which the antiserum used for the therapeutic preparation was obtained. This antiserum, however, by the mouse protection test had only 1 or 2 units per cubic centimeter protection against the freshly isolated Type III strain, "Schultz," which was used for one of the series of intradermal infection tests reported below. The Type III "Schultz" strain was recovered from the lung of a postmortem case February 17, 1930. The three other Type III strains used to produce infection in rabbits were "R_3," the stock laboratory strain, "Cartell," a strain isolated from the meninges December 19, 1929, and "Challenger" isolated from the blood of a fatal case April 9, 1930. A comparative study of the development of the skin lesion and bacteremia and the therapeutic treatment with antisera was made using the Type III strains and a Type I strain.

Method

The cultures were grown in beef heart broth (pH 7.5) to which small amounts of sterile defibrinated horse blood had been added. All cultures were of such a virulence that 0.000,000,01 cc. of an 18 hour standardized culture given intraperitoneally killed mice in 48 to 72 hours. The virulence was tested by injecting the high dilutions of the culture into mice about ½ hour before the intradermal injection was made in the rabbits.

Healthy gray rabbits, male or female, weighing 1525 to 2750 gm. were selected. The abdominal area of each rabbit was shaved and depilated the day previous

[4] *Jour. Exp. Med.*, 1928, **48**, 1 and 413.
Jour. Immunology, 1929, **17**, 279.

to the inoculation. Inoculation was made 6 to 7 cm. lateral to the rabbit's ventral midline. Each animal received an intradermal injection of 0.001, 0.0005 or 0.0002 cc. of pneumococcus culture.

Eighteen hours after inoculation, 1 cc. of blood was withdrawn from the marginal vein of the ear, by means of a sterile syringe, and expelled into a tube containing 1 cc. of 2 per cent citrated beef heart broth. Previous experiments had proved that 2 per cent sodium citrate in rabbit blood, the smallest amount that could be depended upon to prevent coagulation, did not very appreciably inhibit the growth of pneumococci. As soon as possible 0.1, 0.3 and 0.6 cc. were pipetted from the citrated blood broth suspension into sterile petri dishes and melted agar, cooled to 45°C., to which 2 per cent horse blood had been added, was poured into the petri dishes. The citrated rabbit blood and agar were gently rotated until the mixture was uniform. The plates were incubated for 24 to 48 hours at 37.5°C. The sum of the colony count of the three plates is approximately the number of diplococci per 0.5 cc. of blood.

The remaining 1 cc. of citrated rabbit blood dilution was added to beef heart blood broth. Occasionally pneumococci will grow in the liquid medium when the poured plates for a given blood culture are negative. Blood broth cultures were made from the heart's blood of animals which died during the course of an experiment, or which were killed at the conclusion of an experiment.

Preliminary experiments were carried out to determine the extent and character of the lesion produced by Type III strains and their power of invading the blood stream (Table I).

A stock strain "R_3" which has been passed through mice at least once a week during its cultivation in the laboratory for many years and a freshly isolated strain, "Cartell," were inoculated intradermally into rabbits. Four rabbits received the "R_3" strain and two rabbits received the "Cartell" strain in 0.001 cc. doses. The stock strain in a dose estimated to contain 130,000 diplococci stimulated lesions of different severity in individual rabbits which lasted from 2 to 10 days. The freshly isolated strain "Cartell," in a dose containing 350,000 diplococci, produced severe lesions lasting until death of the animals. Later, because of the early death of the rabbits in the first experiment, the "Cartell" strain, after preliminary passage through a rabbit, was injected into six rabbits in duplicate doses of 0.001, 0.0005 and 0.0002 cc. One of the animals which received 0.001 cc., 120,000 organisms, had a slight lesion lasting only 3 days. The other developed a lesion of moderate severity which lasted until its death on the ninth day. One of the rabbits inoculated with one half as many diplococci, 0.0005 cc., developed a severe lesion and died on the sixth day. The other rabbit had a slight lesion on the second day and survived. The rabbits which received 0.0002 cc., 24,000 organisms, developed moderate lesions which lasted in one instance until the death of the animal on the tenth day and in the other more than 14 days.

The Type III lesion was found to be definitely established at 18 to 48 hours as a slightly swollen area 1 to 5 cm. in diameter, regardless of whether the strain used was a stock or freshly isolated one. The redness and edema increased to 3 to 10 cm. with parts of the surface of the lesion hemorrhagic after 2 to 3 days. In our experiments Type III strains did not produce necrosis, nor did the lesions tend to spread extensively over the abdomens of the rabbits. The development of the lesion in rabbits inoculated with Type I culture was much more rapid than in those inoculated with Type III. Redness and edema of 5 to 10 cm. with hemorrhage was not unusual 24 hours after intradermal inoculation. Necrosis with seepage of exudate at central area of lesion was pronounced in 95 per cent of the untreated Type I rabbits in 48 to 72 hours.

The comparative power of the different Type III strains to invade the blood stream was as follows: The stock strain used in four rabbits was not found in the blood stream over a period of 13 days. Three of the four animals survived. One rabbit died on the seventh day but no pneumococci were recovered from the heart's blood at autopsy. On the twenty-eighth day the remaining animals were killed and autopsies made. No pneumococci were found in cultures made from the heart's blood of these animals. The freshly isolated strain "Cartell," inoculated into two rabbits, caused their death within 48 hours. Plate counts of the blood of these animals 18 hours after inoculation showed 186 pneumococcus colonies per cubic centimeter in one and in the other, numbers too great to count in the amount examined. Pneumococci were recovered from the heart's blood at autopsy.

The results with the rabbits inoculated with Type III, "Cartell," in doses of 0.001, 0.0005 and 0.0002 cc. were as follows: one of the rabbits inoculated with 0.001 cc. culture, actually one-third the number of organisms injected in the first "Cartell" experiment, died after a bacteremia of 8 days duration. The other rabbit had a positive blood culture on the sixth, seventh and eighth day and died on the ninth day. It is interesting that pneumococci were recovered as late as the fourteenth day from the blood stream of two rabbits which survived after the inoculation with 0.0005 cc. and with 0.0002 cc. culture. In two instances pneumococci were found to be present in the blood in 24 hours after injection. In one rabbit pneumococci were found after

48 hours, in another after 72 hours and in a third after 96 hours. Two of these rabbits died. The rabbit which survived had over 1660 organisms per cubic centimeter at 72 hours, 126 organisms per cubic centimeter at 96 hours and later four negative blood cultures with subsequent occurrence of small numbers of organisms in the blood for 5 days.

The invasive power of a Type I stock strain inoculated intradermally in a dose of 0.001 cc. into eight rabbits is given for comparison (Table II) with the results with Type III. Pneumococci were recovered from the blood stream of all the rabbits 18 hours after the intradermal injection of the culture. The dose of diplococci introduced into the rabbits varied from 130,000 to 400,000 but there was no correlation between the severity of lesion or the day of death and the number of diplococci injected. All animals died in 3 to 6 days after inoculation of the culture. Pneumococci were recovered from the heart's blood at autopsy.

The effect of treatment of rabbits infected with Type III pneumococci with Type III antiserum was investigated (Table III).

The freshly isolated "Cartell" strain, which was used in the experiments reported above, and a still more recently isolated Type III strain, "Schultz" were used for these tests. 0.001 cc. of these strains was inoculated intradermally into two series of rabbits. All animals responded with definite lesions and bacteremia in 18 hours. Two animals of each series received no antiserum and served as controls and the other four received a single intravenous injection of Type III antiserum 24 hours after the intradermal inoculation of the cultures. Relatively large amounts of serum were tried because the results in mice led us to believe that in Type III infections large amounts would be necessary. Those inoculated with the "Schultz" strain were protected by 20 cc. of serum, 20,000 units, and the blood cultures remained negative after treatment. The blood cultures of the rabbit receiving 10 cc., 10,000 units, were found to be negative after treatment and until after the death of the animal when pneumococci were found in the heart's blood cultures at autopsy. The rabbit receiving 5000 units died 1 day after treatment. No pneumococci however, were recovered from the heart's blood at autopsy. It is probable that this rabbit did not die because of the failure of the serum to protect but from some other cause. Of the first series of rabbits injected with the "Cartell" strain, the blood cultures of those receiving 10,000 and 20,000 units were found negative after treatment and the animals survived. The blood of the rabbit receiving 5000 units was found positive on the first day, negative on the second day, 24 hours after treatment and positive again on the third day; but

TABLE I

Development of Local Lesion and Bacteremia in Rabbits Inoculated Intradermally with Stock or Freshly Isolated Type III Strains

Strain and number of diplococci in dose	Dose	Rabbit number		Degree of lesion development and number of pneumococci per cc. in blood after the following days											Survival or day of death	Blood cultures at autopsy
				1	2	3	4	6	7	8	9	10	11	13	14	
	cc.															
R_3	0.001	6-84	Lesion	+	++	+++	++	++								7	Negative
Stock			Pneumo	0	0	0	0	0									
130,000	0.001	6-76	Lesion	+++	+++	+++	+++	+++	+++	++	+	−	−	−		S	Negative
			Pneumo	0	0	0	0	0	0	0	0	0	0	0			
	0.001	6-14	Lesion	++	++	−	−	−	−	−	−	−	−	−		S	Negative
			Pneumo	0	0	0	0	0	0	0	0	0	0	0			
	0.001	6-51	Lesion	+++	+++	+++	+++	++	++	++	++	+	−	−		S	Negative
			Pneumo	0	0	0	0	0	0	0	0	0	0	0			
"Cartell"	0.001	6-92	Lesion	+++												2	Positive
350,000			Pneumo	∞													
	0.001	6-83	Lesion	+++	+++											2	Positive
			Pneumo	186													
"Cartell"	0.001	3-73	Lesion	+	+	+	++	++	+++	+++						9	Positive
120,000			Pneumo	0	0	0	0	42	26	68							
	0.001	27	Lesion	+	+	+	−	−	−	−						8	Positive
			Pneumo	∞	∞	∞	∞	6,000	226	170							
"Cartell"	0.0005	29	Lesion	+	++++	++++	++++									6	Positive
60,000			Pneumo	0	0	0‡	430										

	0.0005	25	Lesion	−	+	−	−	−	−	−	−	−	−	−	−	S	Negative
			Pneumo	0	0	1,660	126	0	0	0	0	40‡	48	16	0‡		
"Cartell" 24,000	0.0002	26	Lesion	+++	++++	++−+	++++	+++	+++	++	++	++	++	+	+	S	Negative
			Pneumo	20	3,500	∞	5,500	540	6	6	74	86	230	80	10*		
	0.0002	24	Lesion	++	++	+++	+++	++	++	++	++					10	Positive
			Pneumo	86	106	100	146	14	6	2	8						

− Negative. + Slight lesion. ++ Fair lesion. +++ Moderate lesion. ++++ Maximum lesion. ∞ Too many to count in the amounts which were plated.

Rabbits receiving 0.001 cc. culture were killed 26 days after initial inoculation of culture.

Rabbits receiving 0.0005 and 0.0002 cc. of culture were killed 27 days after initial inoculation of culture.

* The blood culture of Rabbit 26 negative after the fourteenth day.

‡ Blood broth positive.

TABLE II

Development of Local Lesion and Bacteremia in Rabbits Inoculated Intradermally with a Type I R1 Strain

No. of diplococci per dose	Rabbit number	Lesion development after the following days						No. of pneumococci in the blood after the following days						Day of death	Blood culture
		1	2	3	4	5	6	1	2	3	4	5	6		
400,000	3-96	+++	++++	++++				120	∞	∞				4	Pos.
	3-71	+++	++++					540	∞					3	Pos.
130,000	6-95	+++	+++	+++				1,500	9,000	∞				4	Pos.
	6-94	+++	+++					410	6,000					3	Pos.
150,000	3-87	+++	+++	+++				1,220	∞	∞				4	Pos.
	3-70	++++	++++	++++				∞	∞	∞				4	Pos.
400,000	11-36	+++	++++	++++	++++	++++	++++	6,300	6,500	180		350		6	Pos.
	11-35	+++	++++	++++	++++	++++		6,200	63,000	100,000				5	Pos.

after that was found negative and the rabbit survived. No end point of protection was found in this experiment.

Another series of rabbits injected intradermally with the "Cartell" strain was treated with smaller doses of serum. The blood cultures of those receiving 5000 and 1000 units were found negative after treatment and the rabbits survived. The blood cultures of one of the rabbits receiving 500 units was positive on first, negative on the second day, 24 hours after treatment, positive again on the third and fourth day and the rabbit survived. The other rabbit which was killed on the tenth day because of a severe diarrhoea had positive blood cultures for 8 days. Apparently the dose of 500 units of serum did not check the infection sufficiently to clear the blood at once of pneumococci but delayed its development long enough to aid the rabbits to recover.

The treatment of two series of rabbits inoculated with another freshly isolated Type III strain, "Challenger," was carried out. The "Challenger" strain was isolated from the blood of a fatal case of pneumonia. In the first series the control rabbits died on the fifth and sixth days. The rabbits receiving 1000 units or more of Type III antiserum survived. The rabbits receiving 300 and 500 units of serum died on the second and the sixth day, respectively. It is uncertain how far the overwhelming bacteremia developed in these last two rabbits before treatment influenced the determination of the end point of protection as all the other rabbits at that time had only a few or no organisms in the blood.

Therefore, we tested another series of rabbits with the purpose of determining the end point of protection, more certainly. Our results in this series were not fully satisfactory as the plates made from the blood of the rabbits to determine the extent of the bacteremia before inoculation were so badly contaminated with *Streptococcus viridans* which was present as a contamination in the horse blood used for the blood agar, as to render it impossible to count the pneumococcus colonies accurately. One of the rabbits receiving 150 units of serum died, the other had a bacteremia for 8 days and recovered. With one exception, all the rabbits which received 300 units or more had no positive blood cultures after treatment and survived; one rabbit receiving 500 units either had an overwhelming bacteremia before treatment or developed it shortly afterwards and died on the fourth day of the infection. Protection occurred in this series of rabbits with a smaller amount of serum than we had expected.

Results obtained with rabbits inoculated with Type I pneumococci and treated with Type I antiserum or concentrated antiserum are given for comparison (Table V).

A series of six rabbits received by intradermal injection 0.001 cc. dose of Type I culture containing 200,000 diplococci. Eighteen hours after inoculation all the rabbits were found to have positive blood cultures. Six hours later four of the rabbits received a single injection of serum containing one of the following amounts:

TABLE III

The Effect in Rabbits of Intravenous Injection of Type III Concentrated Antiserum Given Twenty-Four Hours after Intradermal Inoculations with Freshly Isolated Type III Strains

Pneumococcus strain and No. of diplococci per dose	Rabbit number	Concentrated Type III serum. Lot 7 (Felton)		Degree of lesion development after the following days								No. of pneumococci per cc. in the blood after the following days								Survival or day of death	Blood cultures at autopsy
		Cc.	Units	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8		
"Schultz"	14	5	5,000	++								54								2	Negative
100,000	8	10	10,000	+	+	+						50	0	0						4	Positive
	13	20	20,000	+	+	++	+++	+++	+++	+++	++	384	0	0	0	0	0	0	0	S	
	93	20	20,000	+	++	++	+++	++	++	+	+	50	0	0	0	0	0	0	0	S	
	12	0	0	+	+++	+++						186	∞							3	Positive
	99	0	0	+	++	++	++					28	16	8	10					5	Positive
"Cartell"	3-95	5	5,000	+	+	+	−	−	−	−	−	66	0	44	0	0	0	0	0	S	
180,000	3-60	10	10,000	++	++	+	−	−	−	−	−	94	0	0	0	0	0	0	0	S	
	3-53	20	20,000	++	−	−	−	−	−	−	−	244	0	0	0	0	0	0	0	S	
	3-72	20	20,000	++	−	−	−	−	−	−	−	38	0	0	0	0	0	0	0	S	
	3-49	0	0	++	+++	+++						10	∞							3	Positive
	3-88	0	0	++	+++	+++						16	∞							3	Positive
"Cartell"	1-00	0.5	500	+	+	+	−	−	−	−	−	28	0	8	4	0	0	0	0	S	
200,000	3-83	0.5	500	++	+++	+++	+++	+++	+++	++	+	40	10	12	6	16	4	2	8	*	Negative
	2-97	1.0	1,000	+	++	+	+	+	+	−	−	14	0	0	0	0	0	0	0	S	
	2-07	1.0	1,000	+	+	−	−	−	−	−	−	16	0	0	0	0	0	0	0	S	
	3-50	5.0	5,000	+	+	+	+	−	−	−	−	16	0	0	0	0	0	0	0	S	
	4-00	5.0	5,000	+	+	−	−	−	−	−	−	24	0	0	0	0	0	0	0	S	
	94	0	0	+	+	+	+	+	+			20	16	34	270	48				6	Positive
	3-94	0	0	++	++	+++						14	207,000	∞						4	Positive

* Animal discharged from series on the tenth day because of diarrhea and snuffles. No pneumococci were recovered from the heart's blood.

TABLE IV

The Effect in Rabbits of Intravenous Inoculation of Type III Concentrated Antiserum Twenty-Four Hours after the Intradermal Injection of Type III Pneumococcus (Challenger Strain)

Pneumococcus strain and No. of diplococci per dose	Rabbit number	Concentrated Type III. Lot 7 (Felton)		Degree of lesion development after the following days							No. of diplococci per cc. in the blood after the following days							Survival or day of death	Blood cultures at autopsy
		Cc.	Units	1	2	4	6	8	10	12	1	2	4	6	8	10	12		
"Challenger" 180,000	4-50	0.3	300	+							∞							2	Positive
	2-00	0.5	500	+	+	−	−				∞	∞	∞	∞				6	Positive
	3-74	1.0	1,000	+	+	−	−	−	−	−	6	0	0	0	0	0	0	S	
	3-86	5.0	5,000	+	+	−	−	−	−	−	0	6	0	0	0	0	0	S	
	4-87	10.0	10,000	+	+	−	−	−	−	−	16	0	0	0	0	0	0	S	
	3-98	20.0	20,000	+	−	−	−	−	−	−	10	0	0	0	0	0	0	S	
	3-70	0	0	++	+++	+++					6	∞	∞					5	Positive
	4-96	0	0	++	+++	+++					0	10	∞	∞				6	Positive
"Challenger" 200,000	60	0.15	150	++	+++	+++	+++	−	−	−	Pos.	900	88,500	5,100	4	0	0	S	
	63	0.15	150	++	+++	+++	+++				Pos.	∞	∞	4,200				7	Positive
	65	0.3	300	++	++	+++	+++	+	+	+	Pos.	0	0	0	0	0	0	S	
	66	0.3	300	+	+++	+++	++	+	+	+	Pos.	0	0	0	0	0	0	S	
	68	0.5	500	+	+	−	−	−	−		Pos.	0	0	0	0	0		11	Negative
	61	0.5	500	+	++						Pos.	∞						4	Positive
	67	1.0	1,000	+	+++	+++	++	+	+		Pos.	0	0	0	0	0		11	Negative
	69	1.0	1,000	+	+	+++	+++	+	+	+	Pos.	0	0	0	0	0	0	S	
	62	0	0	+++	+++	+++	+++	+			Pos.	∞	∞	6,000	20			9	Positive
	64	0	0	++							Pos.							2	Positive

TABLE V

The Effect in Rabbits of Intravenous Injection of Type I Antiserum Given Twenty-Four Hours after Intradermal Inoculation of a Type I Pneumococcus Strain

Pneumococcus strain and No. of diplococci per dose	Rabbit number	Type I antiserum[1]		Degree of lesion and development after the following days							No. of pneumococci per cc. in the blood after the following days							Survival or day of death	Blood cultures at autopsy
		Cc.	Units	1	2	3	4	5	6	7	1	2	3	4	5	6	7		
Type I R 1 200,000	6-34	1.0	150	+	++	++					800	832	2,400					4	Positive
	6-49	2.0	300	++	+++	++++	++++		+++		120	0	150	0		0		S	
	6-70	4.0	600	++	++++	++++	+++				400	0	0					4[2]	Positive
	6-57	8.0	1,200	+++	++++	++++	++++		++		500	0	0	0		0		S	
	6-78	0	0	+++	++++	++++	+++				200	3,000	200,000	100,000				4	Positive
	6-69	0	0	+++	++++	++++	++++				1,200	5,000	100,000					4	Positive
Type I R 1 200,000	6-92	2.0	300	++	+++		++++	++++			500	600		800	1,000			6	Positive
	6-89	2.0	300	+	++		+++	++++			1,000	500		250	100			6	Positive
	6-58	4.0	600	+++	+++		++++	++++	++++	+++	300	0		0	0	0	0	S	
	6-32	4.0	600	+++	+++		+++	+++	+++	+++	5,000	500		500	0	0	0	S	
	6-66	0	0	+++							1,000							2	Positive
	6-73	0	0	+++							600							2	Positive
Type I R 1 150,000	82	0.1	150	+++	++++	++++		++++	++++		980	600			0	0		7	Positive
	2-87	0.2	300	+++	++++	++++		+++	+++	+++	1,400	0			0	0	0	S	
	5-06	0.5	750	++	+++	++++		++++	+++	++	2,000	0			0	0	0	S	
	11-37	0	0	+++	++++	++++		++++			2,800	800						5	Positive
	15-54	0	0	++++	++++	++++		++++			50,000	100,000						5	Positive

[1] The first series of rabbits was treated with regular therapeutic Serum 7A, the second with experimental therapeutic Serum K, and the third with refined, concentrated Serum 23-868 Banzhaf.

[2] Death due to injury to heart in bleeding.

150 units, 300 units, 600 units or 1200 units. The rabbit receiving 150 units like the control rabbits died on the fourth day. The rabbits receiving 300 and 1200 units survived. The one receiving 600 units had two negative blood cultures after treatment but died on the fourth day after an injury to the heart inflicted in making a bleeding. Pneumococci were recovered from the heart's blood.

Another series of six rabbits after inoculation with a Type I culture were injected 24 hours later with Type I antiserum as follows: two of the animals received 2 cc., 300 units of antiserum, and two received 4 cc., 600 units of antiserum. The other two rabbits were held for controls. The animals developed slight to moderate lesions within 18 hours after the inoculation of the culture. The blood of one of the rabbits receiving 300 units of antiserum, was estimated to contain 500 diplococci per cubic centimeter at 18 hours; the bacteremia continued to progress until the sixth day, at which time the animal died. Pneumococci were recovered from the heart's blood at autopsy. The other rabbit receiving 2 cc., 300 units of antiserum, developed an initial bacterial count of 1000 diplococci, but there was a gradual decrease in numbers each day until the fifth day only 100 colonies were estimated to be present in 1 cc. of blood. The rabbit died on the sixth day. Pneumococci were recovered from the heart's blood at autopsy. The antiserum given in a 4 cc. dose sterilized the blood of one rabbit, while the blood of the other remained positive for 72 hours before becoming sterile. Both animals survived the pneumococcus infection.

Another group of five rabbits was inoculated with the Type I stock strain and treated with Type I concentrated antiserum. They received intradermal inoculation of 0.001 cc. of Type I culture, which was estimated to contain 150,000 diplococci per cubic centimeter and 24 hours later intravenous injection of a single dose of the following amounts of the concentrated antiserum: 0.1 cc., 150 units; 0.2 cc., 300 units or 0.5 cc., 750 units. A 0.1 cc. dose of the preparation, 150 units, was not sufficient to neutralize the pneumococcus infection in a rabbit which died the seventh day of the disease. A culture made from the heart's blood at autopsy was positive. In rabbits receiving 0.2 and 0.5 cc. doses the bacteremia was checked after treatment. Both rabbits survived the infection.

The control animals of both the Type I series just discussed developed maximum lesions and a progressive bacteremia and died in 2 to 5 days.

DISCUSSION

The virulence of freshly isolated Type III strains for rabbits was apparently somewhat less than that of Type I as shown by the slighter lesion and the later development of the bacteremia. However, all the untreated rabbits, eighteen in number, inoculated with Type III in doses of 100,000 diplococci or more, died. Two of the rabbits inoculated with smaller doses survived. If these are excluded, and only those taken into account which received 0.001 cc., the amount given

to the animals which were treated, the mortality with this dose was 100 per cent. If the rabbits receiving the smaller amounts are included, twenty rabbits of twenty-two died making a mortality of 90 per cent.

Although the number of rabbits treated is too few to allow a close comparison of the relative amounts of serum required, the results indicate that the amounts of Type III serum required to protect rabbits injected intradermally with freshly isolated Type III cultures were not appreciably, if any, greater than the quantity of Type I serum necessary to protect rabbits inoculated with Type I culture. As Type III apparently is slightly less virulent for rabbits than Type I and it has been found by clinical observation that Type III is more virulent for man than Type I, it is not possible to draw inferences from these experiments as to the relative amounts of serum required for treatment of Type I and Type III pneumonia in man.

In spite of this it has been encouraging to find Type III serum potent for protecting rabbits against infection with freshly isolated Type III strains because it was so little effective in protecting mice against these strains. The Type III antiserum used in the above experiments had by the mouse protection tests only 1 to 10 units of protection against freshly isolated strains and 100 to 1000 times as much serum was required to protect mice inoculated intraperitoneally with these strains as with the fully virulent (see above) stock strain. Clinical trial has indicated that Type I therapeutic serum should have a potency somewhat higher than the early Hygienic Laboratory standard (100 units per cubic centimeter) and preferably should have 500 units or more per cubic centimeter. It is reasonable to believe that Type III therapeutic serum should be of equal or greater potency than Type I. If the potency standards in mouse protection units for Type I sera were applicable to Type III, then the Type III therapeutic sera having only 1 to 25 units protection per cubic centimeter against freshly isolated Type III cultures would be of little use. However, while the mouse protection test probably furnishes a fairly satisfactory basis for comparing the relative potency of sera of the same type, it is doubtful whether it is an adequate basis for comparing the strength of the sera of one type with those of another for treatment of pneumonia in man or in animals other than the

mouse. Our experiments in treating intradermal pneumonia in rabbits support this view. It is probable that the suitable dosage of homologous serum for pneumonias caused by each type will have to be determined by clinical trial of serum standardized arbitrarily in mouse protection units as to its relative potency.

Unless it is shown that the determination of the strength of sera by titration of their protective effect in the intradermal pneumonia of rabbits gives more accurate or additional information as to their therapeutic value, we believe the method to be impractical as a routine method for standardization because it is more laborious and more expensive than the mouse method.

SUMMARY

Intradermal lesions and subsequent bacteremia were produced in rabbits by the intradermal inoculation of Type III strains freshly isolated from pneumonia cases. Slight intradermal lesions were produced but no bacteremia was found in rabbits inoculated with an old stock Type III strain.

The intradermal lesions and the bacteremia produced by the inoculation of Type III cultures in rabbits were usually less severe than those produced by the inoculation of Type I culture.

The bacteremia in rabbits was checked and the rabbits protected by suitable amounts of Type III concentrated antiserum given intravenously 24 hours after the infecting dose.

The amount of Type III antiserum necessary to protect rabbits inoculated intradermally with virulent Type III cultures was not appreciably greater than the amount of Type I antiserum required to protect against Type I.

FURTHER EXPERIMENTS IN TYPHUS FEVER

IV. Infection with Washed Mexican Rickettsiae and Immunity to European Typhus

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Plate 39

(Received for publication, August 18, 1930)

In a preceding (1) communication we have reported upon experiments in which we succeeded, in a certain number of rats treated with benzol, in obtaining large numbers of *Rickettsiae* of the Mooser type in the peritoneum as well as in the tunica and in many organs in which *Rickettsiae* had not been previously observed. In some of these rats we found extra-cellular *Rickettsiae* in sufficient number to permit us to wash them, by centrifugation in Locke's solution, and to utilize them for infection free of plasma or cells, or, at any rate, with these elements reduced to such an extent that they could not be held responsible for subsequent symptoms in the infected animals. These previously reported experiments showed that, with such washed organisms, the typical Mexican disease could be produced.

In the present communication we wish to report upon a repetition of such experiments and the subsequent inoculation with European typhus virus of animals that had sustained a typical Mexican disease after infection with the washed organisms.

On June 20, several rats received subcutaneously 2 cc. of equal parts of benzol and olive oil. 2 days later these rats were intraperitoneally infected with tunica scrapings from a guinea pig that had been kept in the cold room, was in its fifth day of disease, showed definite swelling and an adherent testicle, and smears of which showed many *Rickettsiae*. On June 27, 5 days after infection, the rats were killed. One of them showed relatively few *Rickettsiae*, but the one used in this experiment had plentiful *Rickettsiae* both in the peritoneum and in the tunica.

The peritoneum was moist, with some exudate, but there was no free fluid. Both tunica and peritoneum were scraped, and the material from each washed out in 15 cc. of Locke's solution. The two materials were then separately handled, as follows:

(A) Peritoneal scrapings washed out in 15 cc. of Locke's solution, centrifuged 5 minutes at high speed, sediment of cells discarded, and supernatant fluid centrifuged for 45 minutes at high speed. The sediment from this on microscopic examination showed many *Rickettsiae*, but no cells were found in two drops thoroughly searched. This was again taken up in 10 cc. of Locke's solution, and centrifuged at high speed for 40 minutes longer. The sediment again showed many *Rickettsiae* and no cells. This sediment was taken up in 8 cc. of Locke's solution and 4 cc. respectively were injected intraperitoneally into Guinea pig 1 and subcutaneously into Guinea pig 2. Assuming that there may have been as much as 0.5 cc. of actual blood plasma in the original peritoneal scrapings, dilution in the several washings with the final injection of half the last 8 cc. of Locke's solution added would mean that any plasma left would have been in total amount not more than 1/600th cc. Since in each case supernatant fluid was carefully pipetted off from the sediment, it is likely that even less than that was present. If cells remained, they were present in very small number, and as a matter of fact we found none.

(B) Tunica scrapings were similarly treated, except that the final sediment was taken up in 5 cc. of Locke's solution instead of 8, but since these 5 cc. were divided between two guinea pigs, the result is the same. In this material there were no whole cells, but there was a small amount of cell detritus. This material was injected in amounts of 2.5 cc. each, intraperitoneally into Guinea pig 3 and subcutaneously into Guinea pig 4.

The results of these injections are represented in the accompanying curves. The intraperitoneally injected animals, Guinea pigs 1 and 3, developed some temperature, Guinea pig 1 reaching 105°F. on the fourth and fifth days and 106° on the seventh day. Only Guinea pig 3 was castrated, because we did not wish to risk losing both animals, but the swelling was entirely characteristic and in Guinea pig 3 typical *Rickettsiae* were found.

The subcutaneously inoculated guinea pigs, 2 and 4, developed no temperature and no swelling—a matter which, with subcutaneous injection of Mexican typhus fever, is not unusual; moreover, the washing process had consumed, from the death of the rat to the injection of the guinea pigs, about 2 hours, during which a certain amount of attenuation in the Locke's solution is conceivable.

So far, then, the experiments showed a complete confirmation of our previous work, in that guinea pigs intraperitoneally injected with washed *Rickettsiae* developed a disease in every way identical with that conveyed by blood, whole tunica scrapings or other infectious material from Mexican typhus fever animals. In regard to the time

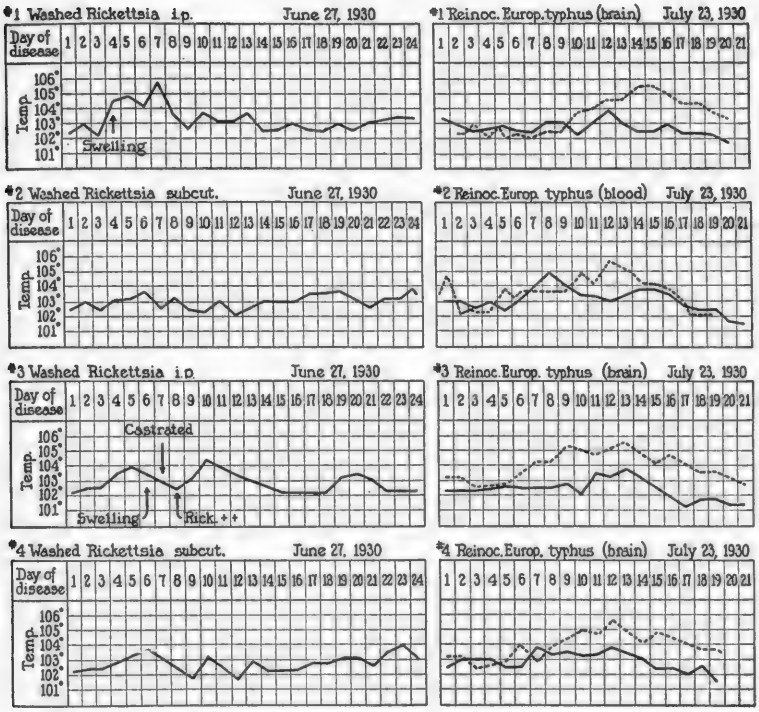
#1 Washed Rickettsia i.p. June 27, 1930
#1 Reinoc. Europ. typhus (brain) July 23, 1930
#2 Washed Rickettsia subcut. June 27, 1930
#2 Reinoc. Europ. typhus (blood) July 23, 1930
#3 Washed Rickettsia i.p. June 27, 1930
#3 Reinoc. Europ. typhus (brain) July 23, 1930
#4 Washed Rickettsia subcut. June 27, 1930
#4 Reinoc. Europ. typhus (brain) July 23, 1930
Day of disease
Temp.
Swelling
Castrated
Rick. + +

CHART 1. The above curves represent the results of the inoculations described in the text. The four on the left are curves of the temperatures of four guinea pigs inoculated with washed *Rickettsiae* of the Mexican variety, Guinea pigs 1 and 3 intraperitoneally, Guinea pigs 2 and 4 subcutaneously injected. The curves on the right are continuations of the same guinea pigs respectively after inoculation on July 23 with virulent material from European typhus animals. In Guinea pigs 1, 3, and 4 the injection was made with brain emulsion, in Guinea pig 2 with citrated blood. In each case, in the curves on the right, the European control animal is shown in broken lines above the continuous line which represents the temperature of the experimental animal. It will be understood of course that every one of the controls is applicable to every one of the experimental animals except in the case of Guinea pig 2, where experimental animal and control were inoculated with blood. Discussion of the interpretation to be applied to this chart is incorporated in the text.

at which temperature and swelling appeared, the disease resembled that produced by whole tunica scrapings, since with blood and organ materials the symptoms are usually deferred for several days. That the subcutaneously inoculated animals did not react is not surprising, since this happens not infrequently when ordinary virus material is injected.

Reinoculation was deferred until July 23 because, in the 4 or 5 days preceding this, there was an extremely hot spell, during which about twenty-five normal guinea pigs, on which temperatures were taken, reached from 103.8° to 105°F., and it was not regarded safe to continue the experiment until the hot weather had abated. It is the heat which we believe accounts for a temporary rise of temperature apparent in the curves of all but one of these guinea pigs on the 21st of July, the date on which the temperatures of the normal controls were taken, the laboratory temperature at that time ranging between 88° and 92°F.

On July 23 the four guinea pigs described were reinoculated intraperitoneally with virus from a European typhus animal on the fifth day of its fever, when the temperature was slightly above 105°F. Guinea pigs 1, 3 and 4 were injected with 2 cc. each of brain suspension. Guinea pig 2 received 4 cc. of citrated heart's blood. Four controls were injected at the same time in a similar manner, three with brain, one with blood.

The results of these inoculations are shown in the accompanying chart. In every case the temperature of a control is drawn in broken lines, the temperature of the previously inoculated animal in a continuous line. They are charted in this way for convenience and in order to save space, since of course all the controls are applicable to each experimental animal. It is apparent from the chart that every single one of the control animals ran the typical temperature curve of European typhus. In every case the temperature reached 104°F. or above between the eighth and the tenth days, remaining above this point for from 6 to 7 days. The experimental animals, on the other hand, showed in no case a typical European typhus curve. The two guinea pigs that had been originally inoculated intraperitoneally with washed *Rickettsiae* and Guinea pig 4, inoculated subcutaneously with washed *Rickettsiae*, could under no circumstances be regarded as having had a European typhus temperature. Guinea pig 2, which had shown no reaction from subcutaneous inoculation with the washed *Rickettsiae*, did develop a sudden rise of temperature on the eighth day, touching 105°F. The temperature came down immediately, however.

We are quite confident in interpreting this rise of temperature in this guinea pig, and the more moderate temperature rise of Guinea pig 1, as due to the hot weather, which returned at about this time. Nevertheless, however rigidly one criticizes the chart, it is quite obvious that Guinea pigs 1, 3 and 4 showed a definite immunity, and Guinea pig 2 either immunity or increased resistance to an amount of European virus which in every one of four controls gave rise to typical European typhus fever.

Incidental to these observations is the interesting fact that the European control guinea pig charted with experimental Guinea pig 3 showed on the fifth day a slight enlargement of the scrotum, without adhesions, and that on castration and examination of the tunica we found in this European typhus animal a few cells filled with *Rickettsiae* indistinguishable from those of the Mooser type found in the usual Mexican typhus animal. This is in corroboration of previously recorded observations by Pinkerton and by ourselves (3), and confirms us in the belief that, in order to find *Rickettsiae* in the tunica vaginalis of European animals, it is necessary to examine early, certainly before the seventh day, since later examinations are almost invariably negative. Moreover, Dr. Pinkerton tells us that it has been his experience in searching for such organisms in the European strain that tunica lesions and *Rickettsiae* are more apt to occur during the hot summer months.

SUMMARY

The experiments recorded in the preceding paragraphs justify, we believe, the following statements:

They confirm our previous observations that *Rickettsiae* of the Mooser type, obtained in considerable numbers in the peritoneal cavities of some benzolized rats and free from cells and plasma to an extent excluding the participation of these substances in any experimental inoculation effects, can produce the typical Mexican disease in guinea pigs.

Animals which have been subjected to infection with such washed *Rickettsia* material prove themselves subsequently either completely immune or highly resistant to inoculation with considerable amounts of European typhus material which produces typical temperature curves in control animals.

The fact that animals which have passed through the disease caused by the washed *Rickettsiae* are subsequently resistant to European tyhus infection justifies a number of interesting conclusions. In the first place, knowing what we do about cross immunization between Mexican and European typhus infection, these experiments show that the disease conveyed by the washed *Rickettsiae* represents the entire picture of Mexican typhus fever in these animals and that it is not necessary, in consequence, to postulate the co-existence of some virus other than the *Rickettsia* in infectious blood, organs or tunica scrapings. In the second place, it seems clear from these experiments that if washed Mexican *Rickettsiae* will immunize to European typhus fever, the etiological agent of European typhus is likely to be a *Rickettsia* organism identical with or closely related to that causing the Mexican disease and, incidentally, the disease observed by Maxcy (4) in the southern United States. The last conclusion is corroborated by our confirmation of Pinkerton's results in finding organisms indistinguishable from the Mooser type of *Rickettsiae* in early tunica lesions of some European animals.

We believe it likely that Tabardillo and the disease of Maxcy are caused by organisms similar to but not identical with those causing European typhus, differing particularly in capacity for selective localization in the body of guinea pigs, the Mexican variety localizing with great regularity and causing violent local reactions in the tunica vaginalis, the European variety localizing without much reaction only during the very early periods of the disease, rarely causing noticeable gross changes and distributing from there to the organs more slowly than is the case in the Mexican guinea pig disease.

Although our present paper does not deal with the particular problem, we would like to add that all our experience with animal inoculation, tissue culture, etc., is against the conception of *Rickettsiae* as true bacteria, since, so far, they have been rigidly dependent for multiplication upon survival of susceptible animals cells, whether insect or mammalian.

CONCLUSIONS

Precise interpretation of our experiments seems to impose the following conclusions:

Guinea pigs inoculated with washed *Rickettsiae* from Mexican typhus fever develop a disease identical with that resulting from inoculations with whole tunica scrapings, blood or other virulent material, and become thereby immunized to European typhus fever.

The etiological agent of Mexican typhus fever is the *Rickettsia* body of the type described by Mooser (5) in the tunica vaginalis of infected guinea pigs; and it is likely that the etiological agent of European typhus fever is an organism similar to this, but not identical with it in some of its minor biological characteristics.

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5. Mooser, H., *Jour. Inf. Dis.*, 1929, **44**, 186.

EXPLANATION OF PLATE 39

FIG. 1. One of two tunica cells seen in smears of a European typhus guinea pig castrated on the fifth day after inoculation, when slight swelling was noted. Only two such cells were found in an extensive search, but these were absolutely characteristic.

FIG. 2. Washed *Rickettsiae*. × 900.

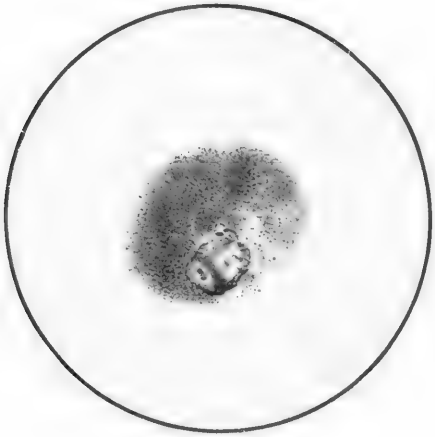

FIG. 1

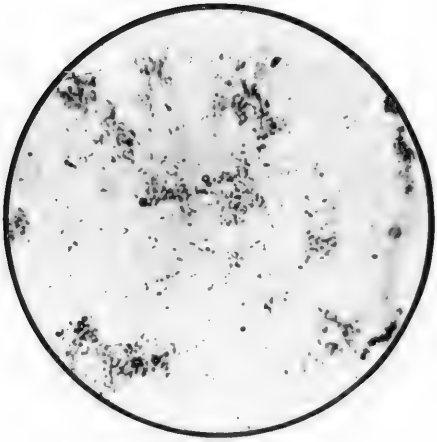

FIG. 2

(Zinsser and Castaneda: Typhus fever. IV)

UNIV. OF MICH.

THE REACTION OF THE ALBINO RAT TO THE INTRA-AURAL ADMINISTRATION OF CERTAIN BACTERIA ASSOCIATED WITH MIDDLE EAR DISEASE

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(Received for publication, August 5, 1930)

Observations on middle ear disease under natural conditions of infection in a colony of albino rats maintained on a balanced diet were previously reported.[1] Adult animals upwards of 1 year in age showed an incidence of 69 per cent, young animals 3 to 4 months in age an incidence of 32 per cent. The bacterial flora of the tympanic cavity was found to embrace a wide variety of microorganisms.[2] Three of these bacteria (*B. actinoides*, a group of streptococci, and a diphtheroid) were encountered sufficiently often with both age groups to suggest that they might be of etiological significance. The high incidence of isolation, however, was the only evidence favoring such a relationship. The particular bacteria might equally well be regarded as secondary invaders. The present paper is concerned with an experimental attempt to define the infective capacity of the three microorganisms for the rat by direct introduction into the tympanic cavity.

EXPERIMENTAL

A disease-free stock of rats was not available for the proposed experiments. Animals from the colony in which the infection was endemic and which had supplied the material for the original study were employed.[3] Young rats between 2 and 3 months of age were chosen in preference to adult animals because of the lower incidence of natural infection. The rats used in any one experiment were generally picked from two or more litters and an approximate balance between

[1] Nelson, J. B., and Gowen, J. W., *J. Infect. Dis.*, 1930, **46**, 53.

[2] Nelson, J. B., *J. Infect. Dis.*, 1930, **46**, 64.

[3] The writer is indebted to Dr. J. W. Gowen for supplying the rats used in these experiments.

males and females was maintained. The natural rate of infection was further reduced by selection at the time of injection. It was earlier observed that the passage of a needle through the tympanic membrane into a normal middle ear cavity could be both felt and heard. In the presence of exudate within the tympanum the membrane was dulled and afforded little resistance to the needle. The characteristic click heard upon puncture of the normal membrane was lacking. Rats which showed such an abnormality upon injection were discarded. This method of selection does not eliminate all cases of natural infection and it occasionally throws out a normal animal. Its application, however, was of unquestioned value in the reduction of affected individuals.

For injection the barrel of a tuberculin syringe fitted with a 24 gauge needle was employed. A short length of rubber hose with a mouthpiece of glass tubing was used instead of the usual plunger. The rats were anesthetized lightly with ether in a closed jar just prior to injection. The needle was passed through the opening of the external auditory meatus generally on the left side, the tympanic membrane punctured, and a small amount of material gently blown into the middle ear cavity. The animals withstood the treatment well. With the experiments to be reported the rats were generally injected in groups of 8 or 16. They were killed 1 week later in most instances, the middle ear cavity exposed, and cultures made. The upper respiratory tract and lungs were examined as routine. Additional cultures were often made from the nasopharynx. Unsealed slanted agar (pH 7.6) enriched with 1 cc. of 50 per cent bovine serum bouillon at the base was used both for growing the bacteria employed in the experiments and for culturing after injection. This medium supports an abundant growth of the rat variety of *B. actinoides* and of the other less particular microorganisms. The fluid portion of the cultures, in undiluted state, was used for injection.

TABLE I

Bilateral Aural Examination Following the Injection of Sterile Medium into the Left Middle Ear

Middle ear examined	Gross condition		Isolation of bacteria	
	No. showing exudate	Percentage incidence	No. showing bacteria	Percentage incidence
Right side	0	0	0	0
Left side	1	6	2	12

The direct introduction of bacteria into the middle ear cavity by way of the external auditory meatus appears to have had little application in animal experimentation. What effect the presence of foreign material might have in the small partially enclosed cavity of the

middle ear was not known. There was a possibility that the medium itself, rich in proteins, might call forth an inflammatory reaction on the part of the host. The first experiment conducted was a control on the method of procedure devised to test this possibility.

Sixteen young rats were injected under ether into the left middle ear cavity with small amounts of sterile 50 per cent bovine serum bouillon. The animals were killed after 1 week. They appeared normal in every way during this period. The middle ear cavities were opened, under precautions for asepsis, and cultures made. The gross pathological and the bacteriological findings are presented in Table I.

The right uninjected middle ear cavities were normal in the gross and sterile in every instance. One rat showed a scanty serous exudate in the left cavity which had received sterile medium. No growth was obtained in culture. The left tympanic cavities were normal in the gross with the other animals. In two instances, however, pure cultures of a non-hemolytic streptococcus were isolated.

In addition to the gross inspection of the middle ear a microscopical examination was also made.

After culturing, the cavity was rinsed with a small volume of saline and the washings examined in both fresh and dry preparations. Washings from the normal uninjected middle ear showed chiefly ciliated and unciliated epithelial cells together with erythrocytes and a very occasional leucocyte. The presence of the latter cells may be attributed to hemorrhage from superficial blood vessels ruptured during washing. Washings from the injected middle ear always showed, in addition, large mononuclear cells of the macrophage type. These were characterized by an abundance of large and small cytoplasmic vacuoles and granules. They varied considerably in number from rat to rat. In some instances there were ten or more cells per microscopic field. The contrast between unstained preparations from injected and uninjected middle ear cavities was very striking. The same cellular reaction was also observed following the intra-aural injection of sterile saline.

Injury to the middle ear as a result of the injection procedure calls forth a low grade inflammatory reaction demonstrable microscopically. The presence of exudate in one case and the isolation of bacteria in the absence of exudate in two instances cannot be definitely accounted for. Repetition of the experiment with smaller numbers of rats resulted in the same microscopic reaction unaccompanied by any gross

change. It may be that an occasional rat responds to the slight injury caused by injection with an inflammatory reaction sufficiently marked to be recognized in the gross. However, it can be safely said that the intra-aural injection of serum bouillon into an etherized rat is not followed by clinical middle ear disease in most instances.

Coincident with the introduction of sterile serum bouillon into the middle ear cavity was a slight outward leakage of the fluid through the punctured tympanic membrane. With the substitution of bacterial cultures for the medium it was important to know whether a detectable amount of culture remained in the cavity.

Three rats were injected into the right tympanum with small amounts of a 5 day old culture of *B. actinoides*. The animals were killed immediately and the middle ear cavities as well as the nasopharynx examined. A small amount of fluid was present in the right middle ears of two animals but absent in that of the third. Microscopic examination of saline washings showed the typical capsular aggregates of *B. actinoides* in each instance. Pure cultures of the organism were likewise obtained. The left uninjected middle ear cavities were normal in the gross and sterile. Washings failed to show the characteristic capsules. *B. actinoides* was not obtained in cultures from the nasopharynx. While the amount of fluid culture actually remaining in the tympanum after direct introduction was small it seemed sufficient for infective purposes. There was no indication of an inward flow of the material injected through the eustachian tube into the nasopharynx.

The results of these control experiments were sufficiently favorable to warrant proceeding with injection of the three bacteria. *B. actinoides* was employed in the first experiment.

Sixteen rats were injected into the left tympanic cavity with small amounts of a 4 day old culture of the organism. The culture was recently isolated from the middle ear of a naturally infected rat. The animals were kept under observation for a week and killed. They appeared normal during this time. The usual examinations were made at autopsy. The gross pathological and the bacteriological findings are presented in detail in Table II and summarized in Table III. The experiment was repeated at intervals with smaller groups of rats and different cultures of the same rodent variety of *B. actinoides*. The data for the total series of rats which received intra-aural injection of the organism are included in the latter table.

Three of the first 16 rats showed exudate in the right middle ear cavity. The exudate was sterile in one case, gave a pure growth of staphylococcus in one, and of a streptococcus in another. Twelve of the rats showed exudate in the left middle ear cavity. With the remainder the left tympanum was normal in the gross and

sterile. *B. actinoides* was isolated in pure culture from 9 of the 12 animals with middle ear exudate. No growth was obtained from one. Two others gave pure cultures of a streptococcus and a staphylococcus respectively. The rats of the additional series showed no gross involvement of the uninjected middle ear. The findings of the left middle ear were much the same as for the first group. The recovery rate of *B. actinoides* was somewhat greater with the second series.

The exudate in the left middle ear cavity was commonly purulent in nature. Microscopically it regularly showed large numbers of polynuclear leucocytes with a few large mononuclear cells. Washings from the injected middle ear in the

TABLE II

Bilateral Aural Examination Following the Injection of B. actinoides into the Left Middle Ear

No. of rat	Gross condition of middle ear		Culture from middle ear	
	Right	Left	Right	Left
1	Normal	Exudate	Sterile	*B. actinoides*
2	"	Normal	"	Sterile
3	"	"	"	"
4	"	Exudate	"	*B. actinoides*
5	Exudate	"	Staphylococcus	"
6	Normal	Normal	Sterile	Sterile
7	"	Exudate	"	*B. actinoides*
8	"	"	"	"
9	"	Normal	"	Sterile
10	"	Exudate	"	Staphylococcus
11	Exudate	"	"	Sterile
12	Normal	"	"	Streptococcus
13	Exudate	"	Streptococcus	*B. actinoides*
14	Normal	"	Sterile	"
15	"	"	"	"
16	"	"	"	"

absence of gross exudate showed chiefly epithelial cells, erythrocytes and the large mononuclear cells previously mentioned. With affected rats the exudate occupied approximately half the space of the middle ear cavity in most instances. Two rats of the additional group showed no gross exudate although *B. actinoides* was recovered in pure culture. Saline washings of the middle ear in both instances showed numerous polynuclear leucocytes. Microscopic examination of exudate from the left cavities, either in stained films or hanging drop preparations, often revealed slender rods and filaments. Similar forms are characteristic of the development of *B. actinoides* under certain artificial conditions of growth. The

organism was recovered in pure culture from such cases. The capsules which develop in fluid cultures of *B. actinoides* were never seen in exudate preparations.

In some instances cultures were also made from other sites. Blood drawn by cardiac puncture was cultured from six injected rats. All of these showed exudate in the left middle ear cavity from which pure cultures of *B. actinoides* were obtained in five instances. The organism was not recovered from the blood in any case. Gross changes were rarely noted in the lungs following the intra-aural administration of *B. actinoides*. Small pieces of lung from rats which showed minute foci of involvement were cultured in a few instances. *B. actinoides* was never recovered. Pulmonary lesions are observed under natural conditions in a few rats of this age. The nasopharynx sometimes showed a considerable amount of mucus but never a purulent exudate. Nasopharyngeal cultures were made from five of the above mentioned rats. A mixed growth of *B. actinoides* and other bacteria, chiefly cocci, was obtained in each instance. None of these rats showed involvement of the opposite middle ear cavity.

TABLE III

Summarized Data on the Intra-Aural Injection of B. actinoides

No. of rats	Middle ear examined	Gross condition		Recovery of B. actinoides	
		No. showing exudate	Percentage incidence	No. of cultures isolated	Percentage incidence
16	Right side	3	18	0	0
	Left side	12	75	9	56
30	Right side	3	10	0	0
	Left side	23	76	20	66

No extensive experiments were conducted to determine how long *B. actinoides* persists in the middle ear cavity of the rat following intra-aural administration. Two injected animals were held for 2 weeks and a third for 3 weeks. The exudate in the middle ear cavity which received *B. actinoides* was scanty with some indication of an attempt at organization. Pure cultures of the organism were obtained in two instances. One of the rats held for 2 weeks gave a mixed growth of *B. actinoides* and a diphtheroid.

The intra-aural injection of streptococci was carried out with two groups of eight rats each at different intervals.

Administration was into the left middle ear. The first group received small amounts of a pure culture of a non-hemolytic streptococcus. The second group

received a mixed suspension containing the same non-hemolytic strain and two strains of hemolytic streptococci. The three cultures were recently isolated from natural cases of middle ear disease. The undiluted fluid portion of 48 hour cultures was employed. The mixed suspension contained equivalent amounts of the three strains. The gross pathological and the bacteriological findings were almost identical for the two groups of rats. The summary for the entire series is presented in Table IV.

Two rats of the first group of eight showed exudate in the right uninjected tympanum. The right cavities were normal with all the rats of the second group.

TABLE IV

Bilateral Aural Examination Following the Injection of Streptococci into the Left Middle Ear

Middle ear examined	Gross condition		Isolation of streptococci	
	No. showing exudate	Percentage incidence	No. of cultures isolated	Percentage incidence
Right side	2	12	0	0
Left side	12	75	2	12

TABLE V

Bilateral Aural Examination Following the Injection of a Diphtheroid into the Left Middle Ear

Middle ear examined	Gross condition		Isolation of diphtheroid	
	No. showing exudate	Percentage incidence	No. of cultures isolated	Percentage incidence
Right side	0	0	0	0
Left side	3	18	0	0

Right middle ear cultures from the entire series were sterile. Six rats of each group showed exudate in the left injected tympanum. A pure culture of a non-hemolytic streptococcus was isolated from the affected middle ear of one rat from the first group. One rat of the second group gave a pure culture of a hemolytic streptococcus and another of a staphylococcus. Left middle ear cultures from the remaining rats were sterile. Nasopharyngeal cultures were not made as streptococci are commonly found on the nasopharyngeal membrane as a part of the normal bacterial flora.

The exudate present in the injected middle ear cavities was commonly scanty in amount and either serous or mucoid in nature. Microscopic examination of

the exudates showed both polynuclear leucocytes and large mononuclear cells as well as epithelial cells and erythrocytes. There was considerable variation in the number and the proportion of the exudative cells from rat to rat. They were never very numerous, at least in comparison with the thicker purulent material commonly found after the injection of *B. actinoides*. Washings from the left middle ear cavity in the absence of gross exudate showed fewer exudative cells, particularly polynuclears.

The intra-aural injection of the diphtheroid was also carried out with two groups of eight rats at different intervals.

One strain of the organism, of recent isolation, was employed throughout. Small amounts of the undiluted fluid portions of 48 hour old cultures were injected into the left middle ear cavities. The animals were killed and autopsied a week later. The gross pathological and the bacteriological findings are summarized in Table V.

The right middle ear cavities were normal and sterile in every instance. Three rats showed exudate in the left injected tympanum. Two of these were from the first group of eight, one from the second. The left cavities of the remaining rats showed no gross involvement. A pure culture of staphylococcus was isolated from the left tympanum of the first affected rat from Group 1. Cultures from the other two were sterile A pure streptococcus culture was also isolated from the left middle ear of one rat in the absence of exudate. Nasopharyngeal cultures were also made from the eight rats of the second group. The diphtheroid was recovered in no instance.

Exudate present in the left middle ear of the first affected rat was definitely purulent, rich in polynuclear leucocytes. With the other animals which showed gross involvement the exudate was scantier and mucoid. Exudative cells were less numerous. Microscopic examination of the saline washings from the left tympanum, in the absence of exudate, showed large mononuclear cells in addition to epithelial cells and erythrocytes. The cells first mentioned varied considerably in number from rat to rat but were never more numerous than in washings made after the injection of sterile medium.

DISCUSSION

The bacteria employed for the experiments (*B. actinoides*, a group of streptococci, and a diphtheroid) were most frequently encountered among a group of nine microorganisms isolated during a study of middle ear disease under natural conditions of infection. The present experiments demonstrate a significant difference in their infective capacity for the albino rat.

Approximately 75 per cent of the rats injected with *B. actinoides*

showed an inflammatory reaction in the middle ear cavity. In most instances this was visible as a purulent exudate, tending to simulate clinical middle ear disease in adult rats. *B. actinoides* was recovered unilaterally in pure culture from about 60 per cent of the animals injected. An occasional rat showed no gross exudate but microscopic examination revealed the presence of polynuclear leucocytes, and *B. actinoides* was recovered in cultures. Twenty-five per cent of the rats showed no specific response to the injected microorganism. In a few instances *B. actinoides* was not recovered from the middle ear in the presence of a gross exudative reaction.

Seventy-five per cent of the rats likewise showed an inflammatory reaction in the middle ear cavity following the injection of streptococci. This was visible grossly as a mucoid or serous exudate which tended to simulate clinical middle ear disease in young rats. Streptococci were rarely isolated from the middle ear at autopsy. The incidence of recovery was about 10 per cent. Twenty-five per cent of the rats showed no specific reaction to the presence of streptococci in the middle ear. In this case, as with the rats of the preceding series, the microscopic reaction could not be distinguished from that following the injection of sterile medium.

Less than 20 per cent of the rats showed gross inflammatory changes in the middle ear after injection of the diphtheroid. The microscopic reaction in the absence of exudate was characterized by the presence of large mononuclear cells and could not be differentiated from the reaction caused by the introduction of serum bouillon. The diphtheroid was never recovered in cultures.

The rats not only showed group differences in their response to the three induced infections but they also displayed individual differences. This latter variability was more apparent with *B. actinoides* than with the other organisms. It was to have been expected in view of the history of the rat colony. The litters of young rats were random selections from a population in which middle ear disease was endemic. The chances were in favor of the inclusion of rats with native resistance reenforced by an acquired immunity of maternal transfer. Furthermore natural infection to the extent of approximately 30 per cent could be expected. This rate was materially reduced by selection at the time of injection. The actual reduction could not be determined

but the low incidence of unilateral middle ear disease, about 10 per cent, for the 32 rats of the first and last series may be taken as a rough index.

B. actinoides and the streptococci may be regarded as potentially capable of inducing middle ear disease in the albino rat. The route of infection was highly artificial in the experiments here described. The findings merely demonstrate the capabilities of the several bacteria upon direct introduction into the middle ear. The natural mode of entrance is probably through the eustachian tube by way of the upper respiratory tract.

B. actinoides appears to be particularly well adapted for development in the middle ear of the rat. It resists for a considerable period of time the marked inflammatory reaction with which the host opposes it. The infective capacity of the streptococcus, insofar as the present strains are concerned, is decidedly less. It is evidently able to establish itself in the middle ear but in most instances quickly succumbs to the defense of the host. The diphtheroid shows little evidence of pathogenicity for the rat. Elimination of the organism is generally effected without calling forth any specific inflammatory reaction. The several exceptions of the present experiments prevent assumption of a complete lack of pathogenicity. It is highly probable, however, that the diphtheroid is a secondary invader in middle ear disease and of no etiological significance.

An analogy between the experimental findings and the findings with natural middle ear disease may be pointed out. Middle ear exudate with naturally infected young rats is often serous or mucoid in nature and frequently sterile. In a previous study 44 per cent of the middle ear cultures from affected young rats were sterile, 12 per cent from adults. Middle ear exudate following the injection of streptococci is of a similar nature and frequently sterile. With naturally infected adult rats the middle ear exudate is often purulent. A fair number of them yield *B. actinoides* in pure or mixed culture (44 per cent). The injection of *B. actinoides* is commonly attended by a purulent aural exudate from which the organism is often recovered (65 per cent). There is a suggestion from this analogy, applicable to the present rat population, that streptococci may be of chief significance in the causation of middle ear disease in young rats, and *B. actinoides*

in adults. The more strictly parasitic nature of *B. actinoides*, implying the necessity of contact for dissemination, as contrasted with the saprophytic tendency of the streptococci which are found in the upper respiratory tract of normal young rats lends some support to this suggestion.

SUMMARY

The infective capacity of three bacteria commonly encountered during a study of natural middle ear disease in a rat colony has been determined by direct intra-aural injection in young rats. One week after the introduction of *B. actinoides* 75 per cent of the rats showed a purulent exudate in the injected middle ear cavity and 65 per cent yielded pure cultures of the organism. With hemolytic and non-hemolytic streptococci 75 per cent showed a serous or mucoid exudate and 12 per cent yielded the organism in culture. With a diphtheroid 18 per cent showed a gross reaction in the middle ear which was sterile in every case.

The experimental findings are discussed in relation to the etiology of middle ear disease.

STREPTOCOCCI IN INFECTIOUS (ATROPHIC) ARTHRITIS AND RHEUMATIC FEVER

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(Received for publication, August 26, 1930)

The isolation of one or another type of streptococcus from the blood and joints of patients with infectious (atrophic) arthritis or rheumatic fever has been reported by a number of observers and has been denied by many more. Within the past two years interest in this question has revived, due, primarily, to the work of Cecil, Nicholls and Stainsby (1, 2, 3) and of Forkner, Shands and Poston (4, 5).

The group first mentioned were able to isolate streptococci from the circulating blood of 61.5 per cent of their cases of infectious arthritis and of these streptococci 83.3 per cent were culturally and biologically identical and were classified as attenuated hemolytic streptococci (2). Furthermore, blood cultures from 58.3 per cent of their cases of rheumatic fever yielded streptococci, of which 94.3 per cent were classified as *Streptococcus viridans* (3). Cultures of the synovial fluids from the two different types of cases frequently yielded streptococci which were culturally and biologically identical with those isolated from the blood stream. Forkner *et al.* were able to obtain streptococci from 22 per cent of the cultures of joint fluids and from 48 per cent of the cultures of regional lymph nodes from cases of chronic arthritis, and of these streptococci 85 and 90 per cent, respectively, were identified as *Streptococcus viridans* (4). In a more recent paper (5), Poston reported positive lymph node cultures from 60 per cent of cases of infectious arthritis and 93 per cent of the organisms grown belonged to the *Streptococcus viridans* group.

Excellent reviews of the literature pertaining to the problem in question are given by the above mentioned authors.

The work reported in this paper was begun in the winter of 1928–29 in an attempt to confirm the presence of streptococci in the blood stream in cases of infectious (atrophic) arthritis. During the winter and spring of 1929–30, the cultural work was enlarged to include joint

fluids and, whenever possible, regional lymph nodes or subcutaneous nodules from cases of either infectious (atrophic) arthritis or rheumatic fever. Owing to the surprising scarcity of patients with joints which contained sufficient fluid to render aspiration feasible, the number of cases included in the second group was extremely small.

EXPERIMENTAL

The patients for this series, in addition to those from the Boston City Hospital, were obtained through the kind cooperation of the medical services of the Massachusetts General, Children's, Robert B. Brigham and Peter Bent Brigham Hospitals and the House of the Good Samaritan.

Blood Cultures (1928–1929)

Method.—The clot method of Cecil, Nicholls and Stainsby (1) for cultures from cases of infectious arthritis was followed as closely as possible. Extreme care was taken in the preparation and the adjustment of the reaction of the media (beef heart infusion broth). All media were sterilized in the autoclave.

50 to 60 cc. of blood were usually taken and duplicate cultures, "A" and "B," were made. Large test-tubes (200 x 25 mm.) were used, in place of bottles, as containers for the media in order to lessen the chance of cracking and breaking due to repeated flaming. In the earlier control cases, the blood clots were allowed to remain with the serum in the icebox for 48 hours before making the cultures. In all the arthritic and rheumatic fever cases the serum was removed within 24 hours. The "A" cultures were subcultured to blood agar pour plates and blood broth every 3 to 5 days, whereas the "B" cultures were transferred only on approximately the 15th and 30th days. When organisms were found in the "A" tube, "B" was subcultured immediately and regularly thereafter. Most of the plates were examined for growth with a hand lens and frequently with a microscope. Many questionable colonies were picked from the plates and subcultured to blood broth. In one small series of cases, a duplicate set of subcultures was incubated anaerobically. In another, subcultures were transferred to fresh blood broth in series on successive days for 6 days each, *i.e.*, first subculture from original, second from first, and so forth.

The results of cultures made from the blood clots and subcultured, over a period of approximately 30 days, are given in Table I. Growth was obtained from 5 "A" cultures and 2 "B" cultures. Among the 35 control cases, growth was found in 6 tubes; whereas, among the 10 cases of chronic arthritis, 11 cases of acute infectious arthritis and 12

TABLE I

Results of Blood Cultures by the Clot Method

Case No.	Diagnosis	Clot cultures A	Clot cultures B	Case No.	Diagnosis	Clot cultures A	Clot cultures B	Case No.	Diagnosis	Clot cultures A	Clot cultures B
1	Arteriosclerosis	0	0	12	Arteriosclerosis	0	0	30	Arteriosclerosis	0	0
2	Rheumatic pericarditis	0	0	13	Carcinoma	20-*S*, *D*	0	35	Hypertrophic arthritis	0	0
3	Arteriosclerosis	*	0	14	Arteriosclerosis	0	0	38	" "	0	0
4	Prostatic obstruction	*	0	15	Hypertension	0	0	42	Myositis	0	0
5	Cervical abscess	0	21-*S*	16	"	0	0	43	Hypertrophic arthritis	0	0
6	Gout	*	0	17	Chronic nephritis	0	0	44	Gonorrheal arthritis	0	0
7	Carcinoma	*	0	18	" "	0	0	45	Hypertrophic arthritis	0	0
8	Arteriosclerosis	*	0	19	Hypertension	5-*S*	0	46	Gonorrheal arthritis	0	0
9	Hypertrophic arthritis	*	0	25	Rheumatic heart disease	0	0	47	Hypertrophic arthritis	0	0
		0	0	26	Carcinoma	0	0	58	" "	0	0
10	Lobar pneumonia	29-*P*	0	27	Gonorrheal arthritis	5-*B*	0	61	No disease	0	0
11	Rheumatic heart disease	8-*S*	0	28	Secondary anemia	0	0	66	Hypertrophic arthritis	0	0
23	Acute infectious arthritis	0	0	22	Chronic infectious arthritis	0	0	20	Rheumatic fever	0	0
24	" " "	0	0	36	" " "	0	16-*D*	21	" "	0	0
29	" " "	0	0	48	" " "	0	0	32	" "	0	0
31	" " "	0	0	49	" " "	0	0			0	0
37	" " "	0	0	50	" " "	0	0	33	" "	0	0
39	" " "	0	0	56	" " "	0	0	34	" "	0	0
40	" " "	0	0	60	" " "	0	0	52	" "	0	0
41	" " "	0	0	63	" " "	0	0	53	" "	0	0
51	" " "	0	0	64	" " "	0	0	54	" "	0	0
57	" " "	0	0	65	" " "	0	0	55	" "	0	0
68	" " "	0	0					59	" "	0	0
								62	" "	0	0
								67	" "	0	0

0 = no growth, * = contaminated by contact with non-sterile blood broth, *B* = Gram-positive bacilli, *D* = diptheroids, *P* = Gram-positive diplobacilli, *S* = staphylococci and the figures represent the day of subculture.

cases of rheumatic fever, growth was found in only one—a case of chronic arthritis. Six "A" cultures, which on the same day came in contact, in the process of subculture, with blood broth which was later proved to be contaminated, were excluded from the series. Staphylococci were found four times, diphtheroids twice and a Gram-positive diplococcus and a Gram-positive diplobacillus once. No cocci growing in chains were found. None of the anaerobic cultures or those incubated in series showed growth.

It should be noted that growth was never obtained from both the "A" and "B" tubes and that more positive cultures were obtained from the tubes that were subcultured every 3 to 5 days than from those that were opened only twice.

Blood, Synovial Fluid and Tissue Cultures (1929–1930)

Method.—Blood cultures were made using the clot method, as outlined by Cecil *et al.* (3) for cultures from cases of rheumatic fever, except that large test-tubes were used as containers instead of bottles. In addition, blood cultures were taken in the ordinary way by adding approximately 5 cc. of blood to flasks containing 100 cc. beef heart infusion broth. Cecil's directions for making double strength beef heart infusion broth and beef heart infusion agar were followed explicitly; the final reaction of all media, after sterilization in the Arnold, was pH 7.6.

The synovial fluids were all obtained from knee-joints. The lymph nodes were, in both cases, large ones over the femoral artery in the groin on the affected side. The rheumatic subcutaneous nodules varied in location and were, as nearly as one could tell, fairly recent in origin. These nodes and nodules were removed in operating rooms under strict aseptic precautions.* They were then macerated in a tissue press (5), and both the fibrous residue and the supernatant fluid were cultured. The synovial fluid and tissue cultures were made in double strength beef heart infusion broth (pH 7.6), prepared according to Cecil (3), and in ascitic fluid-dextrose-beef infusion agar (pH 7.4) contained in Smith-Noguchi tubes, as described by Poston (5). By far the majority of cultures were made at least in duplicate.

Subcultures from the broth tubes were made at 3 to 7 day intervals to blood broth and to streak or pour plates of blood agar, the "A" cultures being transferred more often than the others. The agar tube cultures were examined with a hand lens at 1 to 3 day intervals. When anything resembling a colony of bacteria

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* We are greatly indebted to Drs. C. C. Lund of the Boston City Hospital, T. H. Lanman of the Children's Hospital and S. G. Jones of the House of the Good Samaritan for surgical advice and assistance.

seemed to appear, the whole culture was removed to a sterile Petri dish. Most of the agar was then ground up in the tissue press and the resulting material transferred to blood broth tubes in amounts of from 1 to 5 cc. In a few instances parallel subcultures were made anaerobically. All the cultures were kept for 45 days before discarding.

The results of these cultures, as summarized in Table II, are most inconsistent.

Five of the 7 blood cultures showed growth in one or more of the tubes or flasks, but at least 50 per cent of all the cultures made from each case proved to be sterile. *Streptococcus viridans* and a nonhemolytic (gamma type) streptococcus were recovered from flask cultures from cases of infectious arthritis and rheumatic fever, respectively. A Gram-positive diplococcus, however, was also recovered with the former and another flask culture and 2 clot cultures showed no growth. Along with the latter a diphtheroid was found and 2 clot cultures were negative.

The agar and blood broth cultures of synovial fluids were all negative with the exception of one agar tube which showed a single colony of a Gram-positive diplococcus on the third day.

The agar cultures of the two lymph nodes showed no growth, but a variety of organisms were obtained from the blood broth cultures.

All the cultures of the subcutaneous nodules from the two cases of rheumatic fever were negative.

The sera from the cases in this series, after aging for several weeks to several months, were tested for agglutinins against two strains of streptococci (B 4 from a case of infectious arthritis and RB 19 from a case of rheumatic fever) obtained through the kindness of Dr. Russell L. Cecil. No significant titers could be demonstrated.

Microscopic Examination of Tissues

Method.—A number of paraffin sections of Zenker-fixed tissue were made and were stained according to the Gram-Weigert method and to Wolbach's modification (6) of the Geimsa technique. These were examined most minutely microscopically using an oil immersion lens and a mechanical stage. Tissues from 3 of the cases in the second series were examined together with those from 4 cases of rheumatic fever which were obtained through the kindness of Dr. F. B. Mallory of the Pathological Laboratory, Boston City Hospital.

TABLE II

Results of Blood, Synovial Fluid and Tissue Cultures

Case No.	Diagnosis	Blood cultures		Synovial fluid cultures		Tissue cultures	
		Clot	Flask	Agar	Broth	Agar	Broth
69	Chronic infectious arthritis	A 0 B 0	A 0 B 8-*SV*, *L*	A 0 B 0 C 0 D 0	A 0 B 0 C 0 D 0		
70	Chronic infectious arthritis	A 0 B 10-*B*		A 0 B 0	A 0 B 0 C 0 D 0		
						Lymph node	
71	Chronic infectious arthritis	A 0 B 0	A 0	A 0 B 0	A 0 B 0	A 0 B 0	A 2-*S* B 0
						Lymph node	
72	Chronic infectious arthritis	A 0 B 0	A 45-*D* B 0	A 0 B 0	A 0 B 0	A 0 B 0	A 6-*D* B 6-*S* C 20-*B*, *L*
73	Chronic infectious arthritis	A 0 B 0 C 0 D 0	A 0 B 0 C 6-*C* D 30-*D*	A 0 B 3-*L*	A 0 B 0		
74	Rheumatic fever	A 0 B 0	A 8-*SN*, *B*	A 0 B 0	A 0		
						Subcutaneous nodule	
75	Rheumatic fever		A 0			A 0 B 0	A 0 B 0 C 0
						Subcutaneous nodule	
76	Rheumatic fever					A 0 B 0	A 0 B 0 C 0

B = Gram-positive bacilli, *C* = Gram-negative bacilli, *D* = diptheroids, *L* = Gram-positive diplococci, *S* = staphylococci, *SN* = gamma type streptococci, *SV* = *Streptococcus viridans* and the figures represent the day of subculture.

As will be seen in Table III, no organisms could be demonstrated in the tissue substance, although all the tissues showed some degree of activity.

DISCUSSION AND SUMMARY

The question of the relationship of streptococci to the etiology of infectious arthritis and of rheumatic fever is of the utmost importance. If a streptococcus or group of streptococci could be shown to be asso-

TABLE III

Results of Microscopic Examination of Tissues

Case No.	Diagnosis	Cultures	Tissue	Histologic pathology	Bacteria found in tissue
71	Chronic infectious arthritis	See Table II	Lymph node	+	None
72	Chronic infectious arthritis	See Table II	Lymph node	++	None
76	Rheumatic fever	See Table II	Subcutaneous nodule	++	None
S-26-1034	Rheumatic fever	None taken	Subcutaneous nodule	++	None
S-29-1045	Rheumatic fever	None taken	Subcutaneous nodule	+++	None
25–99	Rheumatic fever	Blood cultures at autopsy negative	Myocardium	++++	None
29–171	Rheumatic fever	Spleen cultures at autopsy negative	Myocardium Mitral valve Aortic valve	++++	None

+ = slight, ++ = moderate, +++ = marked and ++++ = very marked.

ciated with either disease, some form of specific treatment might be available. The possibility of primary streptococcic infection as the cause of rheumatic fever, and, to a less extent, of acute infectious arthritis would seem to be a reasonable conjecture because of the frequency of associated throat, sinus or other focal infection. To consider that these same streptococci remain in or about the affected joint and to such an extent that they are found in the blood stream in cases of chronic infectious arthritis of years' duration demands a rather unique conception.

Recent investigative work has certainly tended to confirm the importance of streptococci in these diseases, but, if all the published reports are considered as a group, one can not help being impressed with the inconsistency and peculiarities of the findings.

In blood cultures from cases of rheumatic fever Clawson (7) recovered *Streptococcus viridans*, Small (8) and Birkhaug (9) non-hemolytic (gamma type) streptococci, and Cecil *et al.* (3) *Streptococcus viridans*, rarely hemolytic and non-hemolytic streptococci. In blood cultures from cases of infectious arthritis Cecil *et al.* (2) recovered attenuated hemolytic streptococci and occasionally *Streptococcus viridans*, non-hemolytic streptococci and diphtheroids and Margolis and Dorsey (10) green-producing and indifferent streptococci and diphtheroids, whereas from synovial fluids and regional lymph nodes Forkner, Shands and Poston (4) and Poston (5) obtained *Streptococcus viridans* and Margolis and Dorsey (11) recovered green-producing and indifferent streptococci and diphtheroids from epiphyseal marrows, bones and synovial membranes. On the other hand, Jordan (12) and Nye and Seegal (13) and the work reported in this paper have failed to confirm these findings.

If a streptococcus is the infective agent, it is difficult to explain why the organisms recovered so consistently by certain groups of investigators are so different and why the findings of other groups are entirely negative.

The chances of contamination, even with the most careful manipulation, are extremely favorable when using a cultural technique which demands subculturing a fluid medium every 3 to 5 days for a period of 4 to 6 weeks. The question arises as to what organisms should be considered as contaminants.

Cultures found to contain *Staphylococcus albus* have never been judged significant. Margolis and Dorsey (11) have excluded Gram-positive bacilli from their series, but have included diphtheroids. Cecil *et al.* (2) reported the recovery of diphtheroids and *Micrococcus zymogenes* from blood cultures which they did not consider contaminated. Jordan (12) recovered short Gram-negative bacilli and Gram-positive bacilli and questioned their importance. The occurrence of such bacteria would appear to be not unlike that of the non-hemolytic (gamma type) streptococci reported by Small (7), Birkhaug (9) and, rarely, by Cecil *et al.* (2) and Margolis and Dorsey (10, 11). In the work reported in this paper and in previously published work (13) with blood cultures from cases of rheumatic fever, staphylococci, Gram-positive bacilli and diplococci and diphtheroids have been isolated from a certain number of the cultures. Since these were found, as a rule, in only 1 of 2 or more cultures from the same blood and since such organisms

occurred about as frequently in cultures from control cases, they have been considered merely contaminants. The findings in the 9 cases of arthritis with positive blood cultures reported by Margolis and Dorsey (10) are certainly far from convincing. These authors found green-producing streptococci in 5 of the 9 cases, but duplicate cultures on the same blood, with one exception, were always negative and the two repeat cultures were negative. Furthermore, in the 3 cases yielding indifferent streptococci in one subculture all subsequent subcultures were negative. In the work reported in this paper a green-producing and a non-hemolytic streptococcus were isolated from blood cultures, but a Gram-positive diplococcus and a Gram-positive bacillus, respectively, were recovered from the same cultures.

A consideration of the above would seem to point to the probability, even certainty, of streptococci occurring in some cultures as contaminants; and the work of Olitsky and Long (14) and Long, Olitsky and Stewart (15) has clearly demonstrated that the air contamination of cultures of ground material with non-hemolytic green-producing streptococci can occur just as easily as with diphtheroids and staphylococci. It is obvious, in such cases, that the types of organisms recovered are dependent on the flora of the air of the laboratory or of the throat of the laboratory worker, and this point may well explain the differences in the cultural findings under consideration. A number of years ago numerous articles (16–19) appeared relative to the bacteriologic flora of lymph nodes, particularly those from cases of Hodgkin's disease, and it is interesting to note that the organisms recovered were quite similar to those which have been recovered from the blood and tissues of cases of arthritis and rheumatic fever, with the exception that the diphtheroids were, at that time, much more in prominence.

Regardless of elaborate serological studies and animal experiments, streptococci must be recovered consistently by several groups of laboratory workers before their etiologic rôle in chronic arthritis and rheumatic fever can be accepted. Duplicate cultures and repeat cultures should yield the same organism in a generous percentage of cases and cultures from cases of other diseases or from normal persons should be negative. Positive cultures from duplicate cultures opened only at the time when the first culture showed growth would make the findings more significant than if the whole series were subcultured every 3 to 5 days.

The work of Cecil, Nicholls and Stainsby on the bacteriology of

the blood and joints in chronic arthritis and rheumatic fever has apparently been carried out most carefully and thoroughly and their results are very consistent and convincing. In spite of attempts to follow their methods in the selection of patients and in cultural technique, the results on the relatively small series of cases which are reported here fail to confirm their findings.

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CHEMICAL AND IMMUNOLOGICAL PROPERTIES OF A SPECIES-SPECIFIC CARBOHYDRATE OF PNEUMOCOCCI

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(Received for publication, September 5, 1930)

The isolation and identification of constituents of bacteria have in many instances disclosed facts of both biological and immunological interest. From investigations of this character the fact has been established that non-protein material of bacterial origin may participate in immunological reactions.

Pick (1) first reported results demonstrating this possibility. He isolated from typhoid bacilli material which failed to give the usual tests for protein but which reacted specifically in antityphoid sera. The residue antigens of Zinsser (2, 3), obtained from a variety of bacteria, were characterized by their non-protein nature. The identification of the type-specific fraction of pneumococci—a non-protein substance—as a polysaccharide established a definite relationship between the chemical constitution and certain biological activities of these organisms (4). Furthermore, the results of investigations based on these chemical studies have defined more clearly the immunological behavior of pneumococci (4). The strict type-specificity of these bacterial sugars in immunological reactions has been established and has been contrasted with the broad species reaction exhibited by pneumococcus protein. Heidelberger's (5) review of the subject contains a complete report of investigations on bacterial polysaccharides. More recently Lancefield (6) has found in hemolytic streptococci a carbohydrate fraction which appears to be a common constituent of these organisms.

The present report contains the results of an investigation of a non-protein substance derived from pneumococci which is chemically and immunologically distinct from both the type-specific capsular polysaccharide and the somatic nucleoprotein. The pneumococcus constituent to be described has been designated Fraction "C," and appears to represent a hitherto unidentified carbohydrate common to the cell body of all R and S forms of pneumococci.

Extraction of the Substance from an R Strain of Pneumococcus

The bacterial cells from 3 liters of broth culture of an R strain derived from Type II Pneumococcus were collected by centrifugation. The bacteria were resuspended in 50 cc. of salt solution and were repeatedly frozen and thawed to break up the bacterial bodies. To this solution of bacteria 0.5 cc. N/1 acetic acid was added, and the mixture was then heated for 10 minutes in a boiling water bath. The tube was cooled and the coagulated protein was separated from the clear supernatant liquid which contained the "C" substance. In this manner the noncoagulable material from 30 to 40 liters of culture was collected. The combined supernatant extract, after neutralization, was finally filtered through a Berkefeld candle and then concentrated *in vacuo* to 50 cc.

TABLE I

Preparation No.	$[\alpha]_D$	Nitrogen	Reducing sugars on hydrolysis	Highest dilution giving a precipitate with Type III antipneumococcus serum*
		per cent	*per cent*	
1†	+22.90	9.40	18.2	1:1,000,000
2	—	5.86	29.5	1:2,000,000
3	+25.0	5.07	30.0	1:2,000,000

* Although Fraction "C" is precipitated by Types I and II antipneumococcus horse sera, Type III serum reacted in highest titre and has been employed as the test serum.

† Preparation 1 was the first preparation made. The material as isolated still gave a faint biuret test. The high percentage of nitrogen in this preparation probably results from accompanying inert proteolytic products.

Preparation of the Substance

The concentrates from 36 liters of bacteria obtained as described above were again acidified with 0.5 cc. of N/1 acetic acid and reheated for 15 minutes at 100°C. A small amount of coagulated protein was thus separated and removed by centrifugation. The clear solution was now treated with 5 volumes of alcohol and, after standing overnight in the icebox, a precipitate settled out which contained all of the so-called "C" substance, together with certain nitrogenous impurities. This precipitate, when redissolved in saline, was found to give a specific precipitin test with antipneumococcus horse serum of Types I, II and III. (A more detailed account of the serological reactions is presented in another part of this report.) The solution of the "C" fraction was found to contain, however, material which gave a positive biuret test, but this impurity was precipitated from solution by making the mixture alkaline with sodium hydroxide, without loss of the serologically reactive substance. The alkaline solution of the "C" fraction, now at a

volume of 50 cc., was reprecipitated by the addition of 5 volumes of alcohol. The material was centrifuged, redissolved in 40 cc. of water and again precipitated from faintly acid solution by alcohol. This procedure was repeated altogether four times. The carbohydrate recovered from the final alcoholic precipitation was dissolved in 15 cc. of water and was cooled to 0°C. To the solution was added 2 cc. of hydrochloric acid (sp. gr. 1.09). A small amount of insoluble inactive material separated from the solution and was centrifuged off. The clear acid solution was now precipitated with five volumes of redistilled alcohol. After standing at 0°C. for 4 hours the "C" substance was separated by centrifugation. It was redissolved in 10 cc. of water and then reprecipitated with alcohol and acid. The final product was washed free from chlorides with 85 per cent alcohol, and was washed finally with redistilled alcohol and ether. The yield was about 65 mg. from 36 liters of broth cultures.

The properties of the various preparations of the "C" substance thus obtained are shown in Table I.

Properties of the "C" Fraction of Pneumococcus

The somatic, species-specific "C" substance as isolated above (Preparations 2 and 3) was found to be an amorphous product soluble in water and insoluble in organic solvents. Its immunological specificity was not impaired by the prolonged action of pepsin at pH 2.0 nor by trypsin at pH 8.1. A solution of the substance (1:300) gave no Millon's test, no biuret test, no xanthoproteic reaction, and no Hopkins-Cole test. Preparation 1 gave a very feeble xanthoproteic reaction, and a very faint ninhydrin test. The solution gave no precipitate with trichloracetic acid, with tungstic acid, with picric acid, nor with sulfosalicylic acid. It did not precipitate on the addition of chloroplatinic acid, copper sulfate, mercury sulfate, nor uranium nitrate. The substance gave a strongly positive Molisch test, and yielded about 30 per cent of reducing sugars on hydrolysis. By treatment with nitrous acid, this substance, like the type-specific polysaccharide of Pneumococcus Type I, was slowly hydrolyzed, with a corresponding loss in immunological activity, and the appearance of reducing sugars. Unfortunately not enough material was available to follow quantitatively the reducing sugars formed, or to determine the phosphorus or sulfur content. Unlike the type-specific polysaccharide of Pneumococcus Type I, the "C" substance contains no amino nitrogen.

On the basis of the chemical data thus far obtained, Fraction "C" appears to be a well defined chemical entity present in pneumococcus cells but distinct from the other fractions. In order to substantiate the validity of this conclusion serological tests were carried out, the results of which may be briefly described as follows: When material prepared according to the method described was mixed with antipneumococcus horse sera of Types I, II and III[1] and incubated, precipitation occurred with each of the three antibacterial sera. A broad reactivity of this character contrasts sharply with the strictly type-specific limitations of the capsular polysaccharides. The tests were repeated employing three different lots of Type I serum, and two of Type II and Type III antisera. In every instance the type-specific polysaccharides reacted only with the homologous antiserum, whereas Fraction "C" was precipitated with each serum, regardless of type. Although these observations are few in number, nevertheless, the results have been sufficiently clear cut to demonstrate serologically the separate identity of the two test substances. Further evidence of this differentiation, apart from the chemical and serological data, may be found in the fact that *non-type-specific R pneumococci contain Fraction "C."* The serological distinction, obtained by the use of antipneumococcus sera of animal origin, has also been observed by employing sera from patients ill with lobar pneumonia. In a separate communication (7), results are presented which demonstrate, during the course of the disease, the independent occurrence of antibodies reactive with three separate constituents of pneumococci.

The "C" substance, on the basis of its broad reactions with antipneumococcus horse sera, is analogous to pneumococcus "nucleoprotein" in that the precipitation of either substance has no type-specific limitations. Since the non-protein Fraction "C" and the so-called nucleoprotein of the cell are so widely different chemically, the similarity in the scope of their serological reactivity indicates that Fraction "C," like the "nucleoprotein," is a common constituent of all pneumococci. Additional evidence in support of this view is brought by the

[1] Types I, II and III antipneumococcus horse sera were obtained through the courtesy of Dr. A. B. Wadsworth from the New York State Health Laborarories, Albany, New York.

fact that a non-protein substance apparently identical with Fraction "C" has been obtained from several strains of both the R and S forms of pneumococci. Material possessing properties similar to the "C" substance has been extracted from two additional R strains—one derived from Type I and the other from Type II Pneumococcus, as well as from Type II and Type III S organisms. These samples were not so highly purified chemically, nor was it determined whether one strain contained more of the substance than another. However, each strain furnished non-protein material which reacted in each of the three types of antipneumococcus horse sera The crude material derived from S cultures by acid and heat extraction contained both the type-specific polysaccharide and the non-type-specific Fraction "C."[2]

In addition to the serological reactions just described a limited number of observations have been made on the toxicity and antigenicity of Fraction "C."

The Toxicity and Antigenicity of Substance "C"

Toxicity.—Mice have been injected intraperitoneally in amounts up to 1 mg. No evidence of toxicity was manifested by the animals nor was purpura produced. For the injection of rabbits preparations of "C" fraction were used which had not been quantitatively analyzed. However, the intravenous injection of 3 cc. of a concentrated extract elicited no toxic symptoms in rabbits.

Antigenicity.—Three rabbits were injected intravenously with concentrated extract. Although the exact content of Fraction "C" was not known, these preparations reacted in high dilutions with antipneumococcus sera. The animals each received 1 cc. of the concentrate daily for 7 days; after a week of rest, daily injections were again given for 7 days; a similar series of injections was repeated a third time. Test bleedings were made on the sixth day after each weekly series of injections. At no time were precipitins for the "C" substance detectable in the sera of the treated rabbits.

[2] After being informed of the above experiments, Drs. Heidelberger and Kendall of the Presbyterian Hospital in this city also encountered the "C" substance as an impurity in their crude Type IV pneumococcus specific polysaccharide, and will shortly publish confirmatory results.

DISCUSSION

The results reported in this communication demonstrate that pneumococci contain a non-protein fraction (Fraction "C") which is distinct from the type-specific polysaccharide. The chemical studies indicate that Fraction "C" does not fall into the category of proteins or their degradation products, but appears to be a nitrogenous polysaccharide analogous in chemical behavior—but not in serological reactivity—to the Type I soluble specific substance. The "C" substance seems to be present in cultures of the organisms in much smaller quantities than the type-specific carbohydrates. The molecule also is probably smaller since it passes through collodion membranes with ease, and through parchment membranes fairly readily. The results of the chemical tests, when taken together with the serological reactions, indicate that Fraction "C" is a common constituent of all pneumococci and apparently is contained within the body of both R and S cells. Chemical analysis and serological reactivity separate it from both the type-specific carbohydrate and the so-called nucleoprotein. In the doses employed, the "C" substance was not toxic for mice or rabbits, nor, under the experimental conditions employed, was it antigenic.

CONCLUSION

Pneumococci contain a non-protein constituent which, on the basis of its chemical and immunological properties, appears to be a carbohydrate distinct from the type-specific carbohydrate and common to the species.

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THE RÔLE OF MICROBIC VIRULENCE, DOSAGE, AND HOST RESISTANCE IN DETERMINING THE SPREAD OF BACTERIAL INFECTIONS AMONG MICE

I. Pasteurella lepiseptica and Pasteurella aviseptica Infections

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(Received for publication, July 25, 1930)

The studies contained in this and the two following papers were made for the purpose of measuring directly the factors responsible for the spread of certain epidemic diseases. The method consisted in the analysis of microbic dosage and virulence and of host resistance in mouse populations during the pre-epidemic, epidemic, and post-epidemic phases of the infections studied. Moreover, tests were made with a view to determining whether an experimental alteration of host-resistance results in a predictable change in the extent and severity of disease. In this paper, studies on Pasteurella infections are reported; studies on a Friedländer-like bacillus infection and *B. enteritidis* infection are described in the second (Ia) and third papers (Ib) respectively.

Technique

The mice constituting each population were assembled in a single, well-ventilated cage, measuring 35 x 35 x 35 cm. in dimensions. This form of housing, by confining the specific bacteria to a limited and definite space, insured a high degree of control of the bacterial dosage factor. Each population in its respective cage was placed on a large table at relatively equal distances from light and heat sources. The routine diet consisting of pasteurized Grade "B" milk plus white bread was given daily at 8:30 to 10:00 a.m. Bedding consisted of wood shavings. The cleaning was done by transferring the mice twice a week to a sterilized cage containing fresh bedding. To each population, two normal mice with identification marks were added daily. A daily census was kept, and the animals found dead were removed at 9:00 a.m. and 5:00 p.m. These stated intervals were ad-

hered to in spite of cannibalism and partial decomposition of the bodies, because regulated removals of the dead presumably affected the numbers of specific bacteria in the cage at definite rather than random periods throughout the day. The carcasses, unless destroyed by the living mice, were autopsied and studied bacteriologically. Routine cultures were made from heart's blood, lungs, spleen, and intestines to suitable agar plates and identified later by cultural and serological tests.

The mice employed in these experiments came from The Rockefeller Institute breeding room. These mice, strain inbred for more than 15 years, have been kept in a special room where constant breeding regulations, daily routine, and dietary regime prevail. The number of deaths in this breeding stock 500 to 800, plus 500 to 800 offspring, averages about one individual per month. All dead animals are examined. The post-mortem tests may show the presence of a Pasteurella-like pneumonia in 1 to 2 pregnant females per year during the cold weather, and *B. enteritidis* infection in 2 to 3 individuals during the winter and in 8 to 10 during July and August. Numerous tests for fecal carriers have revealed the presence of *B. enteritidis* in less than 0.05 per cent. The mice used in the following experiments were approximately 12 weeks old, and weighed 16 to 18 gm. each.

The Spread of Pasteurella Infections

The first observations dealt with the primary response of a population to certain foreign bacilli. Experiment 1 was made to test the mode of spread of such strains of pathogenic Pasteurella in communities of mice previously unexposed to these organisms.

Experiment 1.—June, 1925, four populations were assembled by placing ten mice in each of four regular cages. Each received a specific culture of Pasteurella. Two fresh mice were added daily to each population.

Population 1, June 27, 1925, received a rabbit Pasteurella strain designated "Rivers G." It was a variant obtained from the "Rivers D" culture by prolonged growth in plain broth, and was relatively avirulent for rabbits (1c). 0.1 cc. of an 18 hour rabbit-blood broth culture was instilled into the nares of each mouse.

Population 2, June 11, 1925, received the fowl Pasteurella strain designated "Pa." It was obtained by Dr. Theobald Smith from an epidemic of fowl cholera and proved highly virulent and relatively non-vegetative for chickens (1d). The bacilli were administered by mixing an 18 hour blood broth culture with bread and placing it in the cage as the food ration for one day.

Population 3, June 27, 1925, received the rabbit Pasteurella strain "Rivers D." This organism was obtained from a rabbit dying of septicemia and represented the highly pathogenic and non-vegetative variety (1c). The mice received an intranasal instillation of 0.1 cc. of an 18 hour blood broth culture.

Population 4, June 27, 1925, was given a similar intranasal instillation of the rabbit Pasteurella strain, DC 30. This culture, obtained from a rabbit dead of

spontaneous pneumonia, is an example of the moderately virulent, vegetative variety common in rabbits (1c).

Text-fig. 1, which tabulates the results of the tests, is constructed as follows: The daily population is plotted as a series of dots; the average survival time of the two mice added on a given day is shown by the solid line, and the numbers of deaths on a given day are indicated by a series of rectangles. These rectangles are marked further to indicate whether or not the animal was autopsied, and if so, what the findings were.

The mortality records raise two questions which should be considered before the general results of the experiments are discussed—namely, the diagnosis and disposal of the few dead mice with negative autopsy findings, and the large number of non-autopsied mice. The following pertinent statements can be made, based on 4 years' data of over 6000 mice. Mice which presented no lesions or specific bacteria at autopsy were for the most part victims of accidental death (fighting, etc.). A few, however, may have died of one of the specific infections and failed to yield the inciting organism. Mice which were not autopsied suffered this neglect because of cannibalism, post mortem decomposition, or lapse in technique. In assigning to these latter a probable cause of death, old age can be ruled out because the known mean age at death of 2000 of these mice is many months less than the known average duration of life of the normal standard mice, and intercurrent infection can likewise be dismissed because none save those infections under investigation occurred. Since 99 per cent of the individuals subjected to autopsy yielded the specific organism, the death of non-autopsied individuals can with safety be attributed to the infections prevailing in the communities at the time. Hence for statistical purposes, all deaths have been considered as specific and in the general discussion, total mortality figures have been employed.

The reactions of mouse populations to the presence of foreign strains of Pasteurella are shown in Text-fig. 1. None of the rabbit strains survived. The "Rivers G" and DC 30 strains in Populations 1 and 4, infected none of the mice and died out at once; the "Rivers D" culture in Population 3 killed 2 of the originally inoculated mice but did not spread. However, the fowl cholera strain, "Pa," in Population 2 behaved in quite a different manner.

Eight of the ten mice originally fed with the "Pa" culture died within 2 weeks with Pasteurella septicemia. During the following 6 weeks, no further deaths from this infection occurred. However, a mouse found dead on July 21 showed Pasteurella "Pa" in blood and organ cultures, together with *B. enteritidis*.[1] This animal had been added to the population on June 27, 5 days after the last Pasteur-

[1] The appearance of *B. enteritidis* in this and other populations is discussed later (1b).

ella fatality. No further Pasteurella deaths were recognized until August 14, 24 days later, when the population numbered 61 individuals. During the succeeding 5 days, however, 47 died. On the first day, 9 succumbed and Pasteurella "Pa" was recovered from the blood and tissues of 5 subjected to autopsy; on the second day, 2 died, also yielding the "Pa" organisms; on the third day, 27 died and from each of the 25 mice autopsied, the "Pa" strain was obtained. Seven mice died on the fourth day, all giving positive cultures, and twelve on the fifth day, four of which were examined and found to contain the "Pa" organism. The epidemic then ceased abruptly and *P. avicida* disappeared from the population.

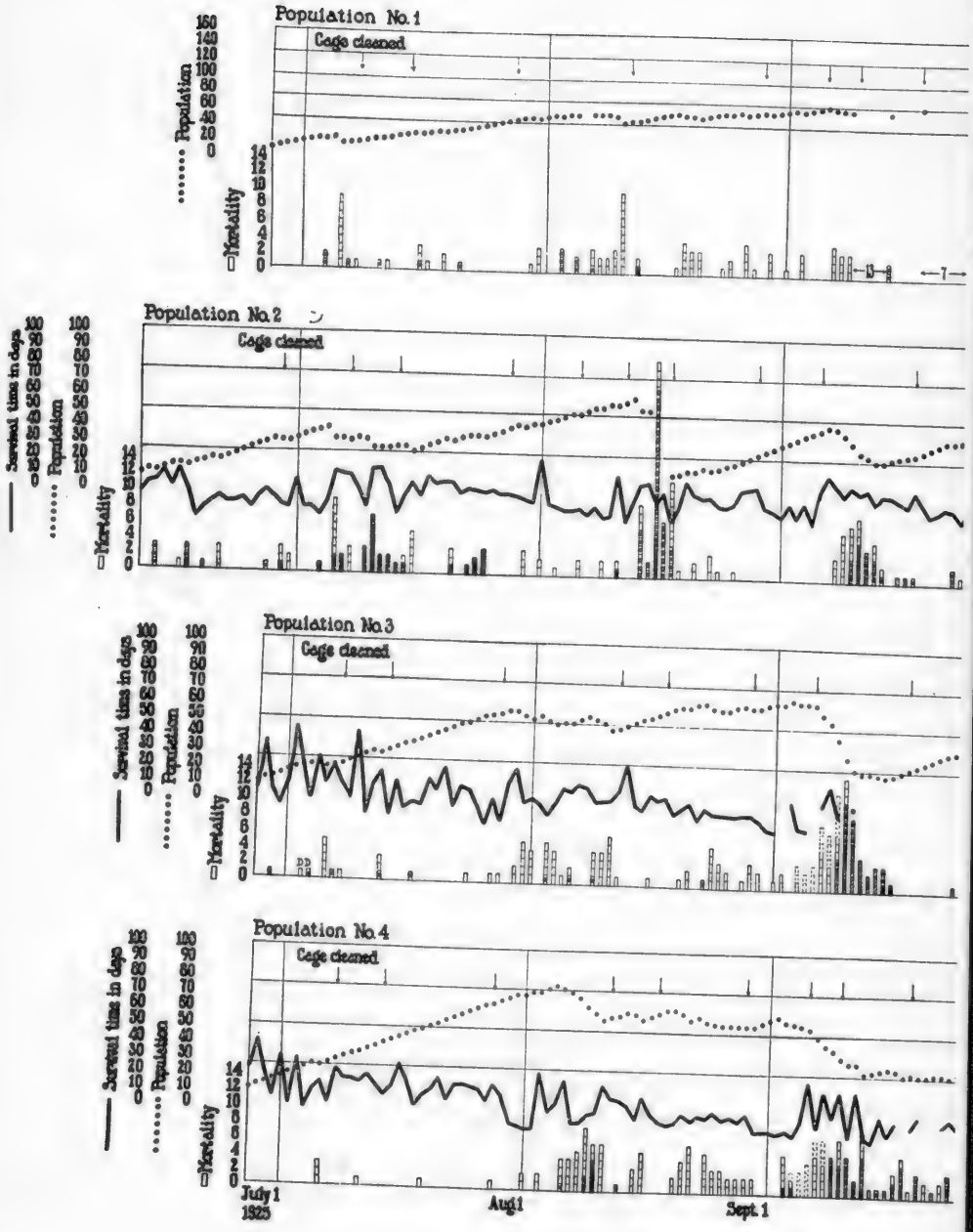
These events demonstrated three facts of epidemiological significance; first, that a "foreign" strain of a highly pathogenic bacterium, *P. avicida*, survived for 2 months in a previously unexposed population without producing obvious injurious effects; second, that after this period of apparent inactivity, the organism led to a severe epidemic fatal in 5 days to 47 mice or 77 per cent of the population; and third, that it failed to survive permanently in the community.

Comparison of Virulence of "Foreign" Strains and "Epidemic" Strains

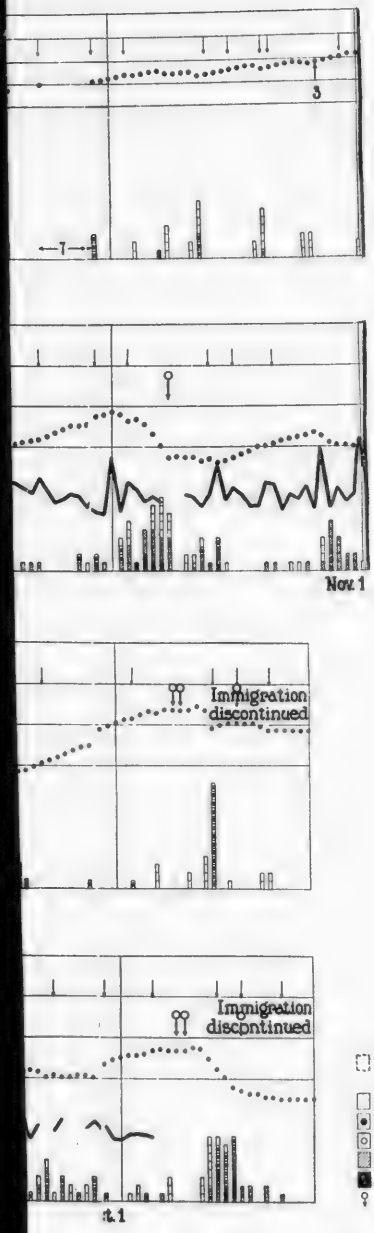
A titration was made to determine whether the virulence of strains recovered at the height of the epidemic differed from that of the foreign strain originally introduced into the community.

Experiment 2.—Heart's blood cultures were taken from six mice which succumbed during the most severe phase of the epidemic, that is three on August 14, one on August 15, and two on August 16 (Text-fig. 1). On August 20, these six "epidemic" strains, together with the original "foreign" strain kept on blood agar and grown 18 hours in rabbit blood broth, were administered to test animals as follows: 0.5 cc. of each culture was given intranasally to two rabbits by instilling 0.25 cc. into each naris; each culture was also inoculated intraperitoneally in 18 mice in saline dilutions 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-9}, 1 cc. of each dilution being given to three mice. The approximate numbers of bacilli administered were determined by plate method.

The results of these titrations are recorded in Table I. The number of organisms in each culture ranged between 2×10^8 and 5.1×10^8; hence effects of similar numbers of organisms of each strain could be compared. These effects were briefly as follows: the two rabbits receiving 2,300,000 bacilli of the original "foreign" strain died in 22.5 ± 1.5 hours, while the twelve receiving similar numbers of the six "epidemic" strains died in an average time of 22.7 ± 1.6 hours. All mice receiving the 10^{-2} dilution of the various cultures—2,000,000–5,100,000 organisms—were dead within 18 hours. The 3 mice receiving 23,000 bacteria of the foreign strain died in an average time of 18 hours, while 18 mice receiving 20,000



TEXT-FIG. 1. Spread of Pasteurella in mouse pop



use poptions. For continuation see Papers II and III.

TABLE I

Comparison of Virulence of "Foreign" and "Epidemic" Cultures of Pasteurella avicida: Strain "Pa"

Culture	Mice												Rabbits	
	Dilution 10^{-2} No. organisms	Survival time Hours	Dilution 10^{-4} No. organisms	Survival time Hours	Dilution 10^{-6} No. organisms	Survival time Hours	Dilution 10^{-7} No. organisms	Survival time Hours	Dilution 10^{-8} No. organisms	Survival time Hours	Dilution 10^{-9} No. organisms	Survival time Hours	No. organisms	Survival time Hours
Stock "Pa"	2,300,000	−18, −18, −18	23,000	−18, −18, −18	230	20, 20, 21	23	39, S, S	3	S, S, S	0	S, S, S	115,000,000	24, 21
10/III/14	2,000,000	−18, −18, −18	20,000	−18, −18, −18	200	0, 21, 23	20	21, 25, 68	2	24, S, S	0	S, S, S	100,000,000	26, 21
5/IV/14	5,000,000	−18, −18, −18	50,000	−18, −18, 22	500	21, 22, 39	50	39, 39, 39	5	S, S, S	0	S, S, S	250,000,000	25, 21
4/V/14	4,000,000	−18, −18, −18	40,000	−18, −18, 20	400	20, 20, 23	40	20, S, S	4	S, S, S	0	S, S, S	200,000,000	24, 21
7/VI/15	5,100,000	−18, −18, −18	51,000	−18, −18, −18	510	21, 39, 68	51	18, 18, 24	5	23, 39, S	0	S, S, S	255,000,000	20, 23
1/VI/16	2,900,000	−18, −18, −18	29,000	−18, 20, 22	290	0, 39, 45	29	39, S, S	3	S, S, S	0	S, S, S	145,000,000	23, 21
3/V/16	3,100,000	−18, −18, −18	31,000	−18, −18, 20	310	−18, 20, 45	31	S, S, S	3	S, S, S	0	S, S, S	155,000,000	23, 25

0 = No animal injected.

S = Alive and well 30 days after injection.

to 51,000 bacilli of the "epidemic" strain died in an average time of 18.7 hours. The average time of death of the mice given 230 organisms of the foreign strain was 20 hours; while the mice given 200 to 510 organisms of the "epidemic" strains succumbed in about 30 hours; a dose of 23 organisms of the foreign strain resulted in 1 death at 39 hours and 2 survivors; similar small doses of the "epidemic" strains led to death in about 33 hours, plus 50 per cent survivors. Few of the 10^{-8} dilution animals and none of those receiving the 10^{-9} dilution died.

According to this titration, the pathogenicity or virulences of the original "foreign" strain and of the 6 "epidemic" strains were similar. It must be noted, however, that the titration was not performed under the best conditions because the rabbit inoculations involved the use of a foreign host and the mouse titrations the use of an unnatural portal of entry. Such a departure from natural methods, in which the native host and normal portal of entry are employed, detracts from the value of the test and furnishes at most presumptive evidence that the highly virulent "Pa" strain of *P. avicida*, gaining access to the mouse community, surviving and spreading with no apparent damage, finally was able to give rise to an epidemic outbreak of great severity without any demonstrable alteration in its disease-producing power.

COMMENT

These observations on Pasteurella infections in mice indicate that the primary response of average unexposed populations to foreign strains of the organisms depends on inherent pathogenicity, or virulence, of the microbes, that the inherent pathogenicity of the bacilli is relatively stable for mice, and that the strain with high killing power possesses low vegetative capacity.

SUMMARY

1. Three strains of Pasteurella native to rabbits, introduced into mouse communities failed to spread or survive.

2. One strain of fowl Pasteurella, so introduced, survived for 2 months without causing death, after which, it gave rise to a 5 day epidemic, fatal to 77 per cent of the population. Subsequently, no further deaths from this source occurred and the strain died out completely.

3. At the height of the epidemic, caused by the fowl strain of Pasteurella, 6 cultures were obtained which, on direct inoculation,

proved to have a degree of virulence for mice and rabbits equal among themselves and the same as the strain originally employed.

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THE RÔLE OF MICROBIC VIRULENCE, DOSAGE, AND HOST RESISTANCE IN DETERMINING THE SPREAD OF BACTERIAL INFECTIONS AMONG MICE

II. B. FRIEDLAENDERI-LIKE INFECTION

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(Received for publication, July 25, 1930)

The studies in the preceding paper (1 a) dealing with the primary response of a community to Pasteurella infection were continued on a more extensive scale with a Friedländer-like bacillus respiratory infection. The disease arose in the mouse populations under observation and gave rise to a number of severe epidemics, during which it became possible to investigate the epidemic factors,—microbic dosage, virulence, and host resistance,—and also to determine the consequence to an infected population of modifying the host resistance factor.

Experimental Material

The Friedländer bacillus disease, as encountered in several mouse populations, has been described previously (1 b) as a highly virulent, respiratory infection, with a portal of entry by way of the nasal passages, incubation period of about 48 hours, and a high mortality. The pathological manifestations consist of a bilateral pneumonia, with or without pleurisy, and scattered, subserous hemorrhages, characteristic of septicemia.

The pertinent facts about the populations in which the Friedländer bacillus disease occurred will be restated briefly. Populations 1, 2, 3, and 4 consisted originally of four groups of ten mice, assembled in four standard cages. Populations 1, 3, and 4 were given rabbit strains of Pasteurella; Population 2, a fowl strain of the same species. Each community was then maintained by the daily addition of two standard mice. The same technique as that previously employed (1 a) was

continued throughout the present studies. Following the administration of Pasteurella, three populations remained uninfected (1 a); the fourth, No. 2, became free of *P. avicida*, after suffering one severe epidemic. *B. enteritidis* gained access to all the communities and persisted throughout the 4 year period of observation, but during this time behaved in such a regular, predictable manner (1 c) as not to interfere essentially with the present studies on the Friedländer bacillus disease.

Occurrence and Spread of the Friedländer-Like Bacillus Infection

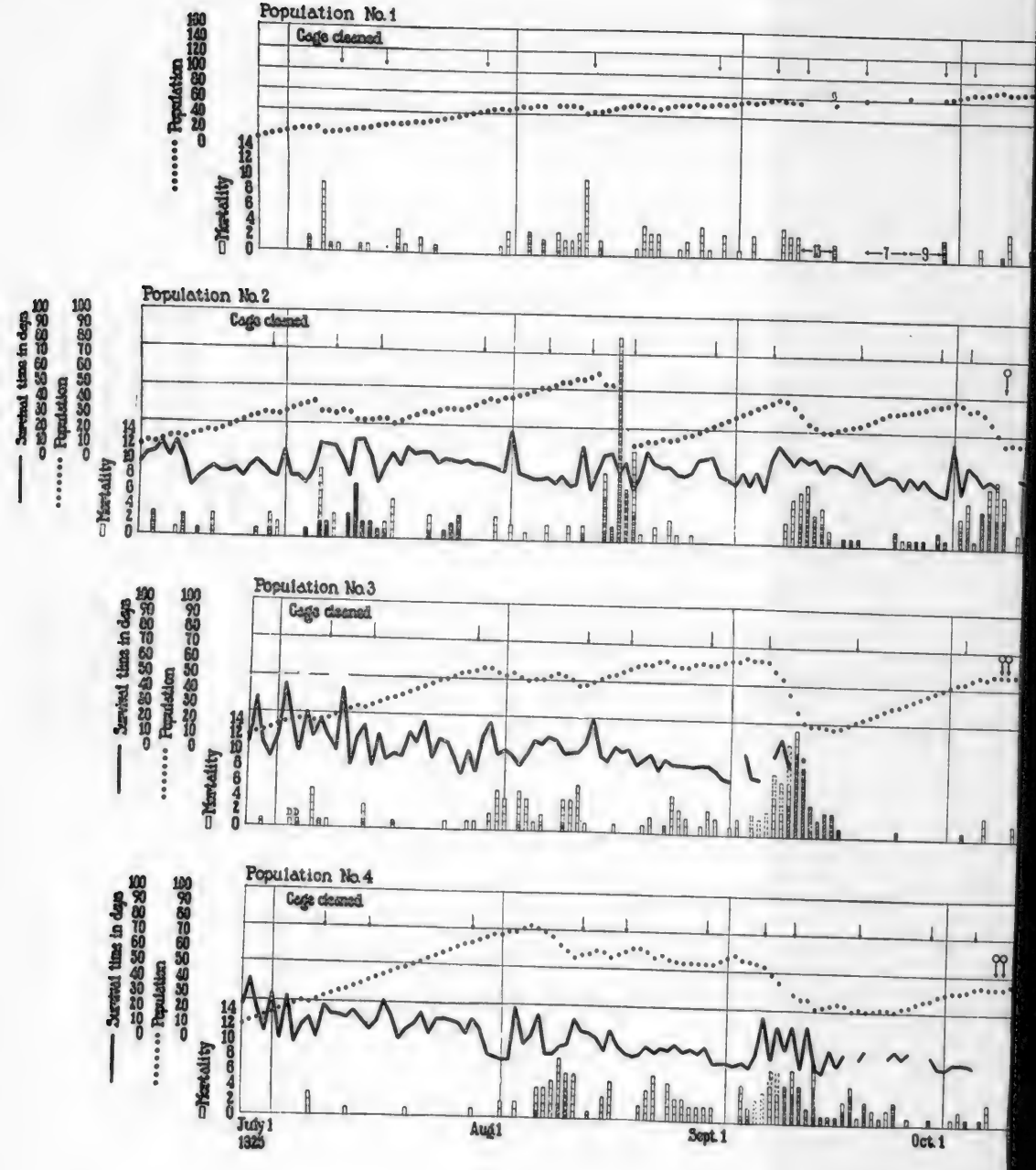
The first appearance of the Friedländer bacillus disease and its subsequent behavior in the several populations took place in the following manner (Text-fig. 1):

Sept., 1925, epidemics of pneumonia arose simultaneously in three (Nos. 2, 3, and 4) of the four mouse populations under observation. Each epidemic was characterized by an explosive onset, a rapid increase of mortality to a peak followed by an abrupt decline, and a subsequent period with very low death rate. The epidemic curves tended to the bell form; more than 50 per cent of each population succumbed. One community, No. 1, escaped entirely.

Following the primary epidemics, the spread of the disease took two distinct courses. In Populations 3 and 4, in which daily introductions of two mice were discontinued, the Friedländer infection died out completely; in Population 2, however, where daily immigrations were continued, the disease prevailed for 5 months. In this community, five secondary outbreaks occurred, four of which followed the primary wave in a regular manner, and the fifth after a 2 months' intervening period of sporadic deaths. After the fifth outbreak in Feb., 1926, deaths from this infection ceased abruptly and the disease seemed to disappear from the community.

In Aug., 1926, however, 1 year after the first outbreak, the Friedländer-like infection reappeared. Previously, for 4 months the censuses of the old populations, Nos. 1 and 2, and of the new one, No. 5, (page 912), were stabilized at about 75 individuals by the regularly occurring deaths from *B. enteritidis* infection (1 c). No evidence of the Friedländer disease was forthcoming until early in August, when it again appeared in each community and destroyed more than 60 per cent of the inhabitants. These epidemics, although similar to those of 1925 in time of onset, differed in their relatively long duration and sequelae.

Following the primary epidemics of 1926, the spread of the Friedländer-like disease assumed two distinct aspects. In Population 1, in which an experimental change in technique was instituted, a definite alteration took place in the type of infection. (This change is described on page 922). In Populations 2 and 5,


Population No. 1
Cage cleaned
Population
Mortality
Population No. 2
Cage cleaned
Survival time in days
Population
Mortality
Population No. 3
Cage cleaned
Survival time in days
Population
Mortality
Population No. 4
Cage cleaned
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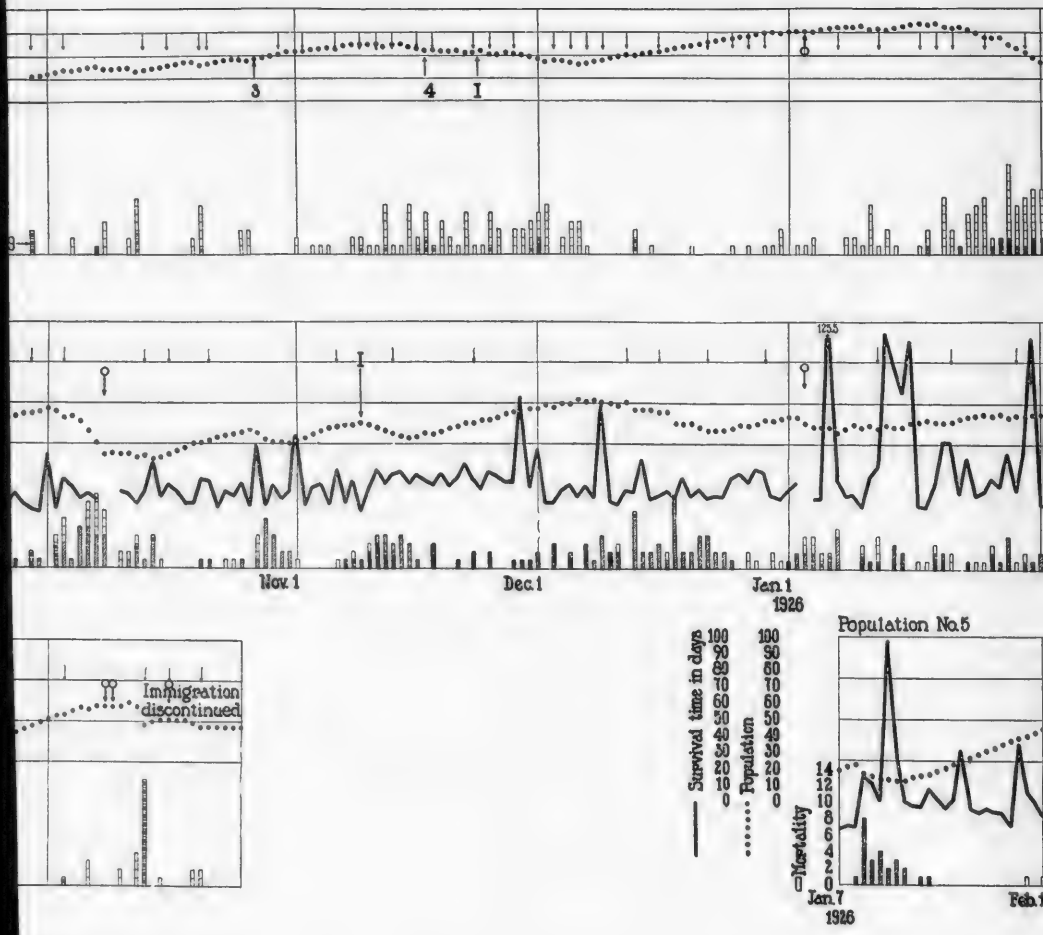
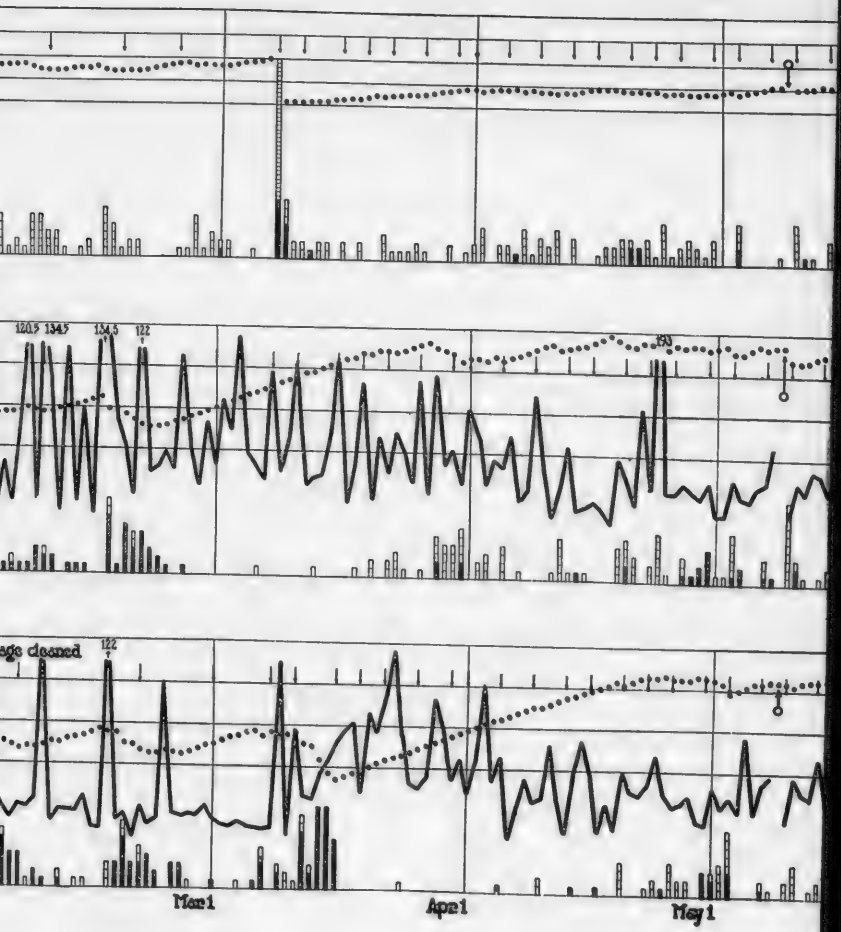
Population
Mortality
July 1
1925
Aug 1
Sept. 1
Oct. 1



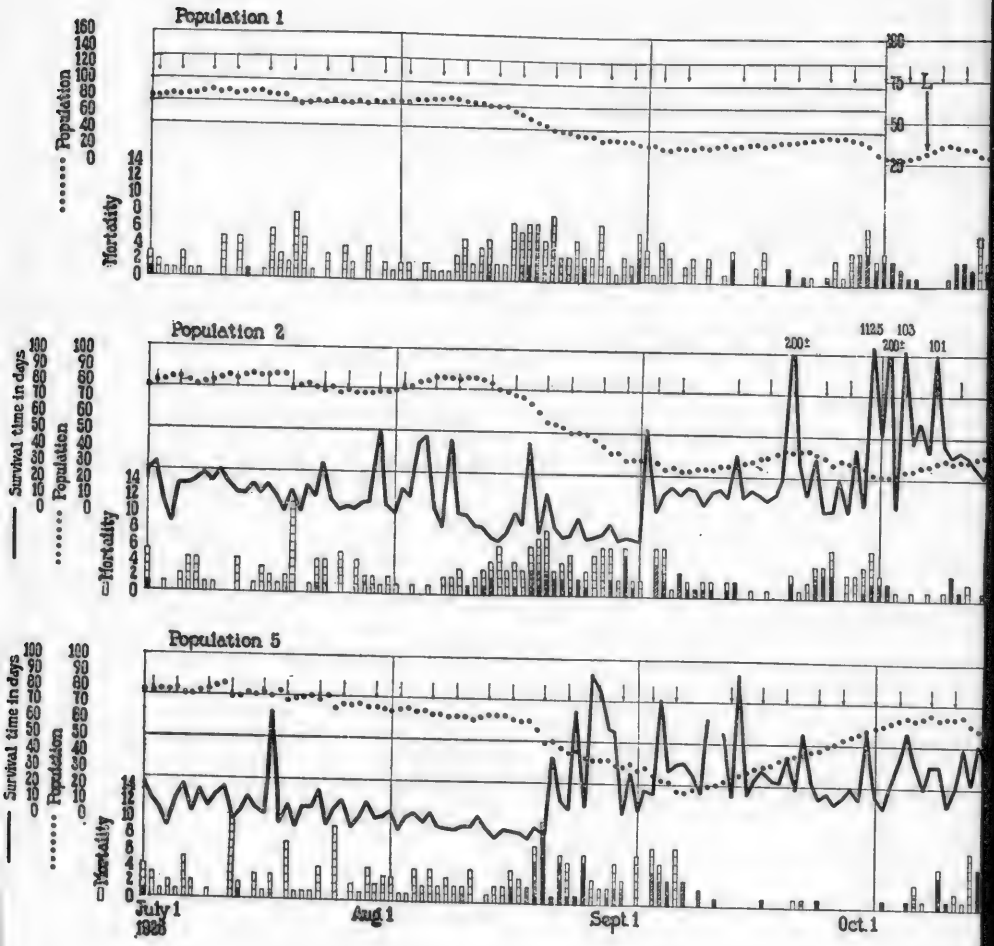
TEXT-FIG. 1*a*

TEXT-FIG. 1, *a*, *b*, and *c*. Spread of Friedländer-like bacillus infection


120.5
134.5
134.5
122
193
age cleaned
122
Mar 1
Apr 1
May 1

Population
Mortality
Survival time in days
Population
Mortality
Survival time in days
Population
Mortality
May 1
June 1
June 30

UNIV. OF MICH.



-1 mouse dead, date of death uncertain within 6 day period

-1 . . . , no autopsy

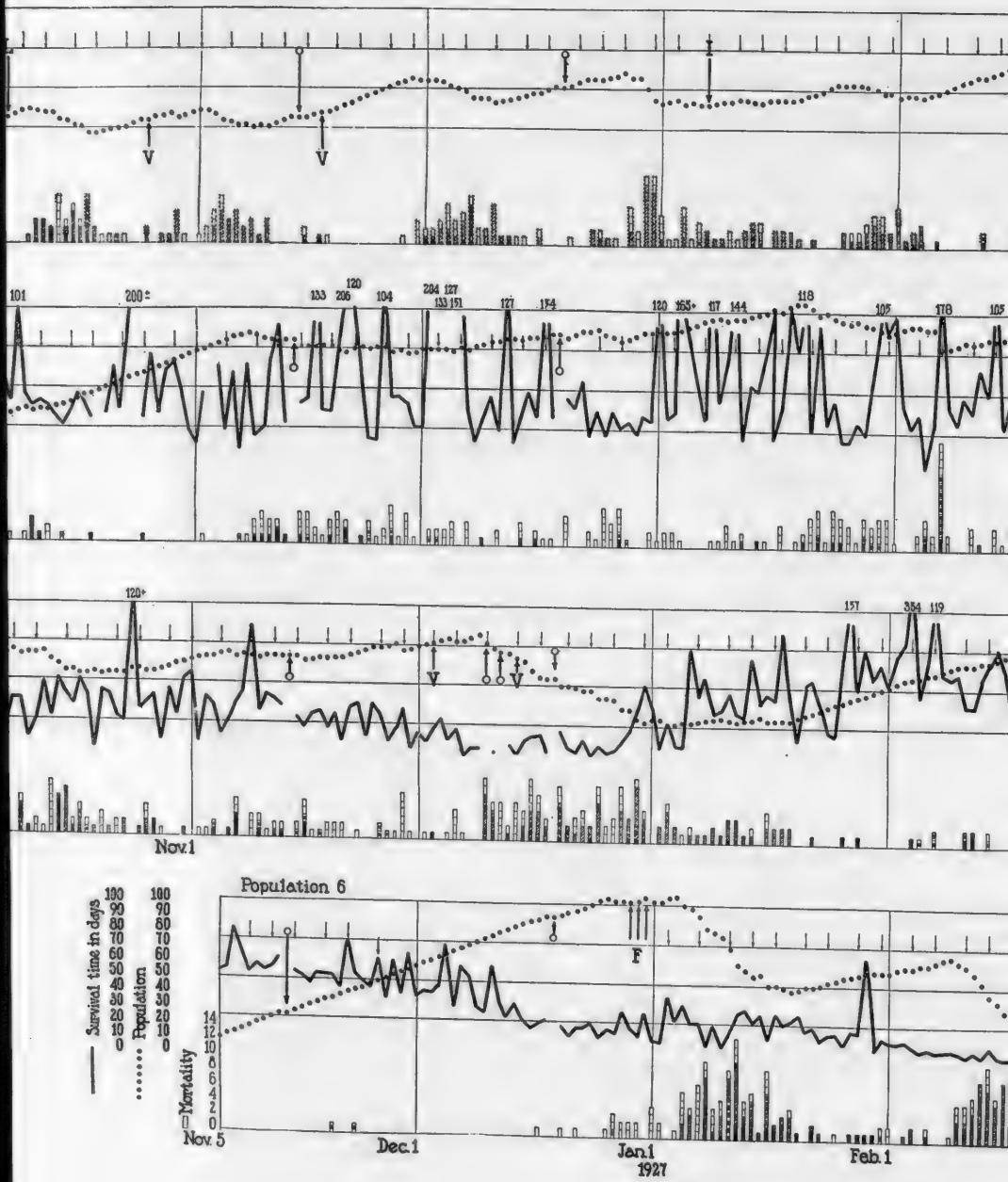
-1 . . . , autopsy findings negative

-1 . . . . . Bact. friedlander

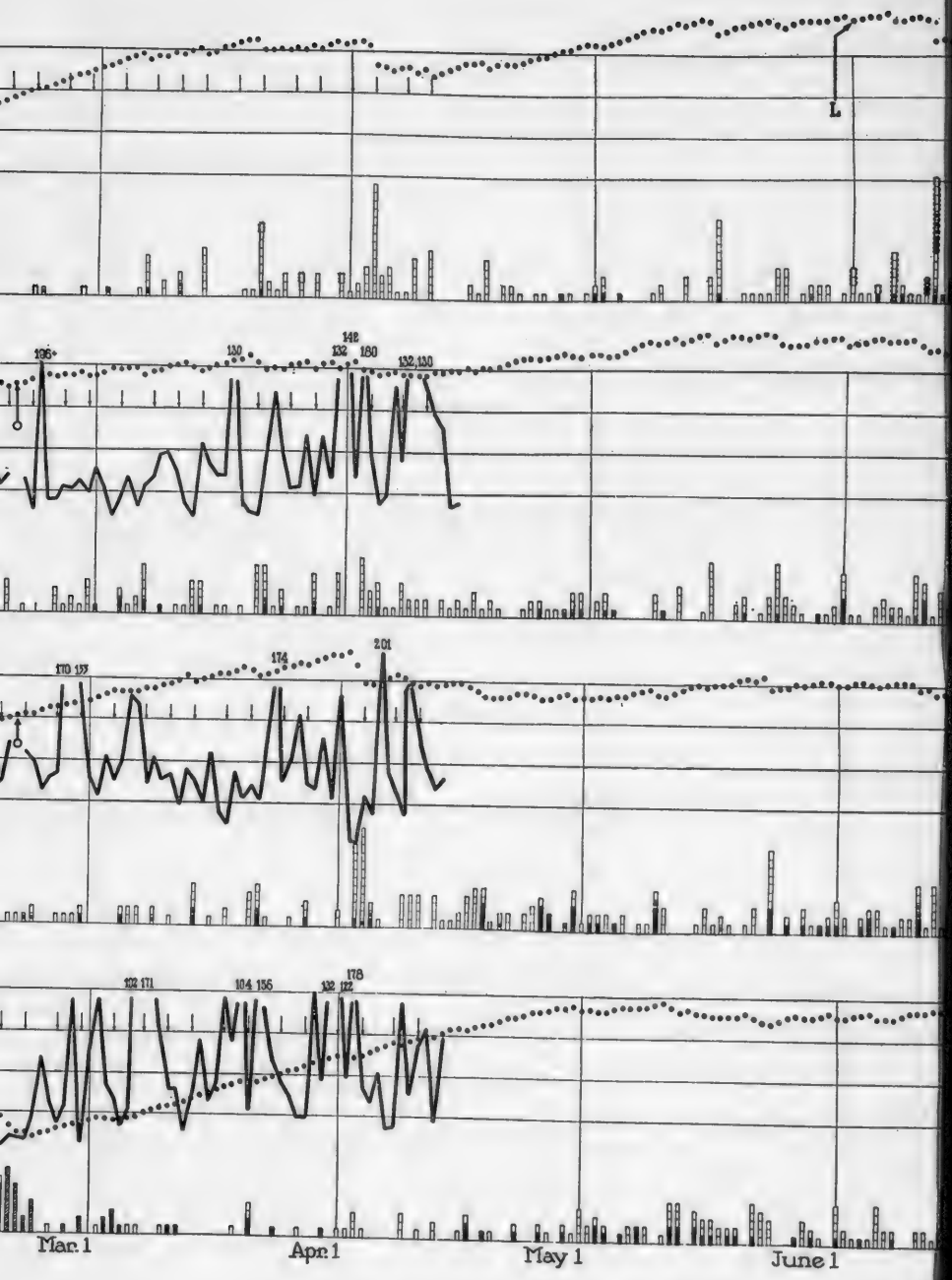
-1 . . . . . B. enteritidis

-Lathrop mouse

♀ -No mice added


V
V
101
200±
133 206
120
104
204 127
133 151
127
154
120 165±
117 144
118
105
178
105
120±
157
354 119
V
V
V
Nov. 1
Survival time in days
Population
Mortality
Population 6
F
Nov. 5
Dec. 1
Jan. 1
1927
Feb. 1

TEXT-FIG. 1*b*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136
130
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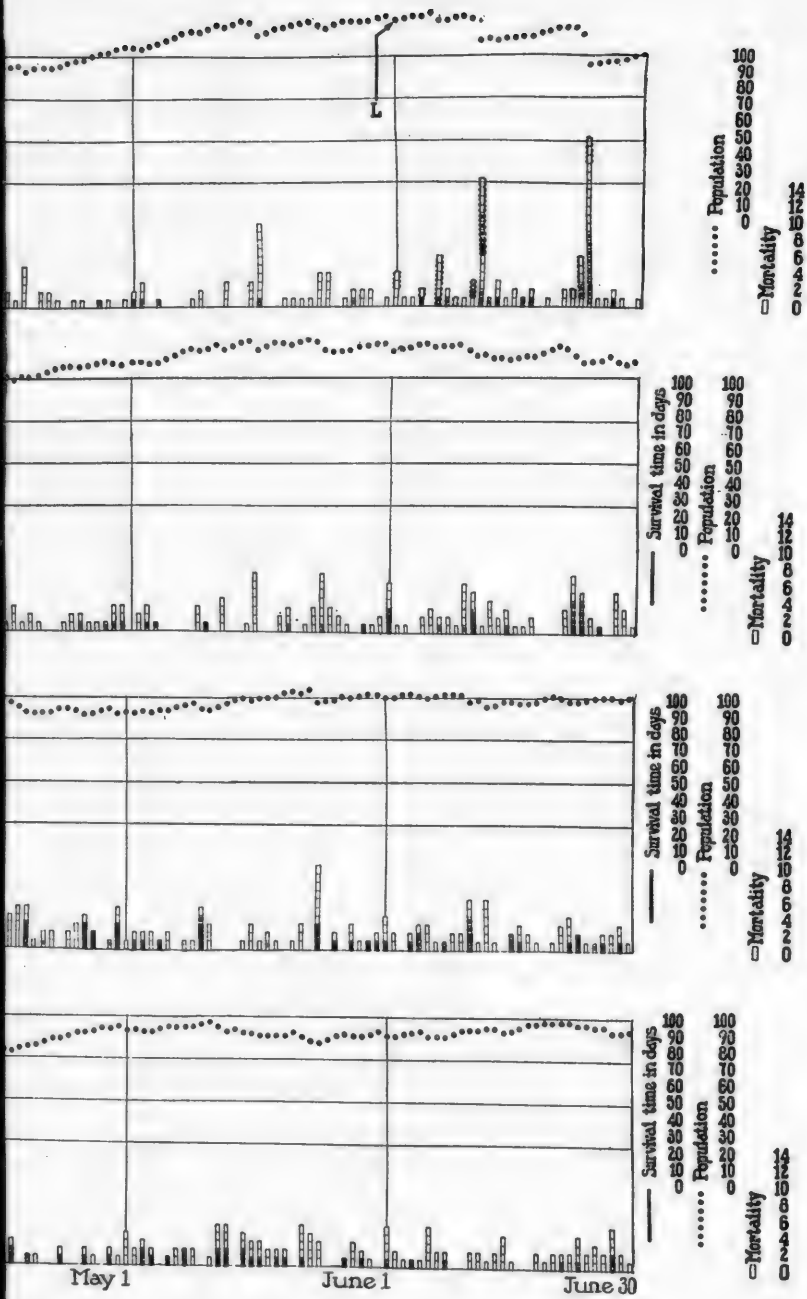
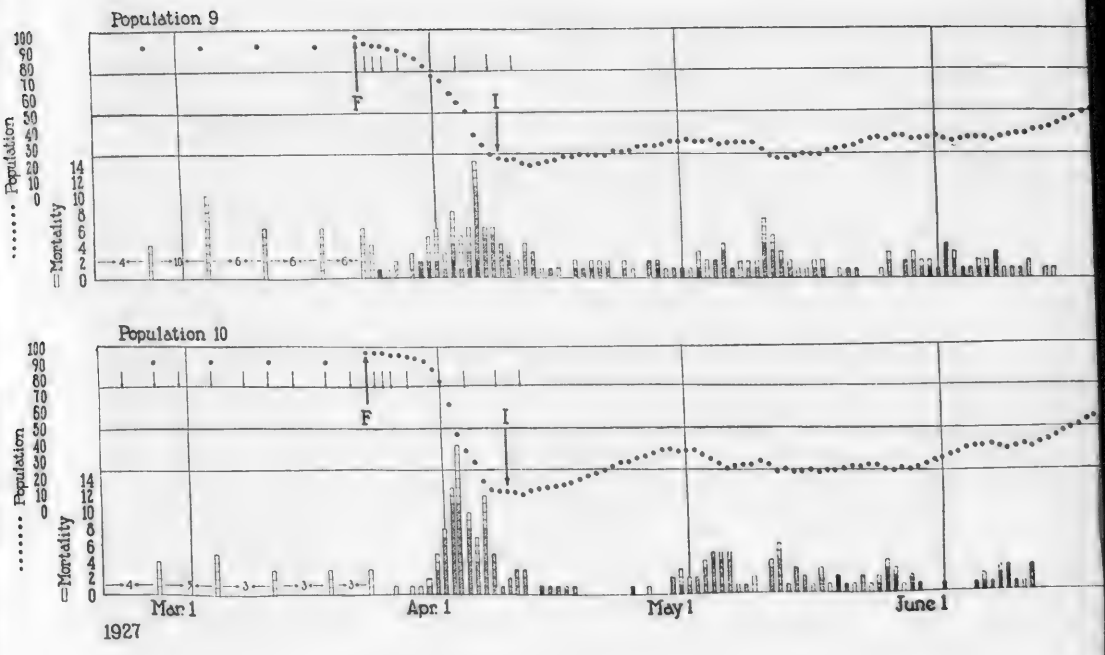
132 122
178
Mar. 1
Apr. 1
May 1
June 1


L
Survival time in days
Population
Mortality
May 1
June 1
June 30



[ ] = 1 mouse dead, date of death uncertain within 6 day period

□ = 1 . . . , no autopsy

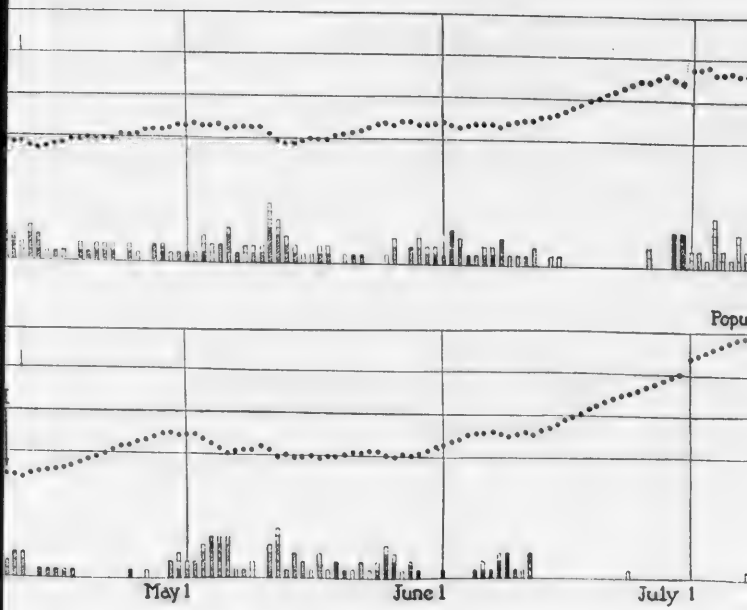
⊙ = 1 . . . , autopsy findings negative

▨ = 1 . . . . . Bact friedlander

■ = 1 . . . . . B.enteritidis

[ ] = Lathrop mouse

♀ = No mice added



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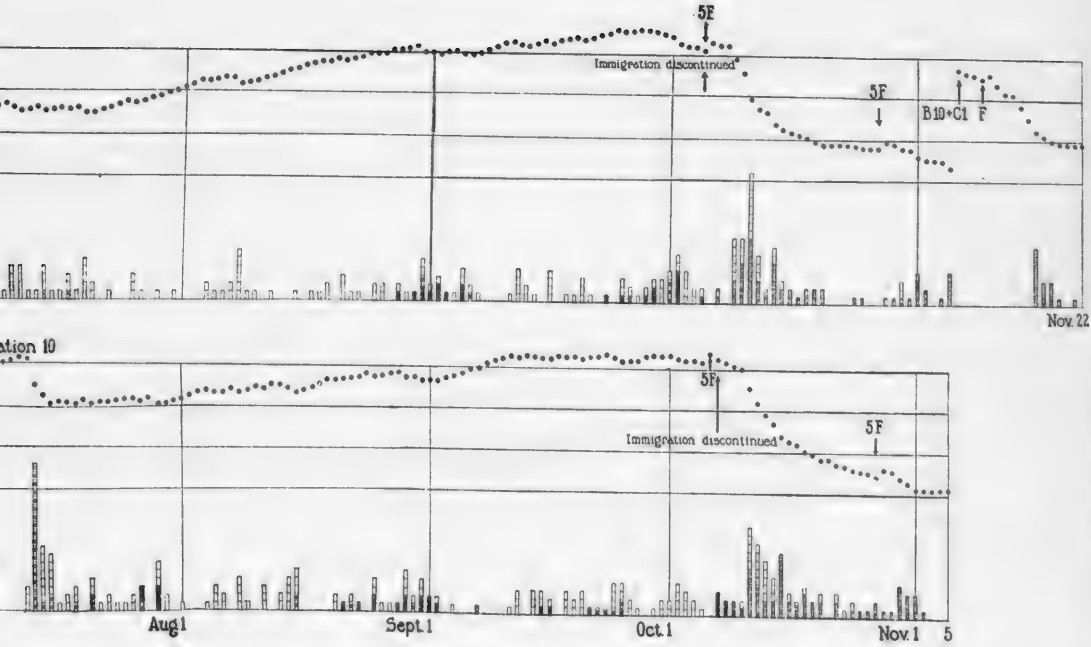
TEXT-FIG. 1c


5F
Immigration discontinued
5F
B10+C1 F
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ation 10
5F
Immigration discontinued
5F
Aug 1
Sept. 1
Oct. 1
Nov. 1
5

however, in which the routine procedures were continued, the infection assumed a relatively mild and chronic form; one secondary wave occurred, followed by a relatively long period with sporadic deaths from Friedländer's bacillus and the complicating *B. enteritidis* infections. Meanwhile the population census increased slowly to 80 individuals, at which time the Friedländer infection disappeared permanently from Population 2 and from Population 5 after one further secondary wave.

In brief, therefore, the epidemiological phenomena to be considered were these: the explosive outbreaks of Friedländer-like bacillus respiratory disease of Sept., 1925, occurring in three communities, the succeeding secondary waves, and later the complete disappearance of the infection; again, the reappearance of the disease during Aug., 1926, in epidemic form, affecting them all, the relatively more chronic nature of these outbreaks, as compared to those of the preceding year, the fewer secondary waves, and finally the permanent disappearance of the disease during the early winter. The source of the original infection was not determined, nor could the manner of its entry into the several populations be ascertained.

Virulence of the Friedländer-Like Microbes

At various occasions during the spread of the Friedländer bacillus infection, the virulence[1] of the specific microbes was tested. These tests were made in two ways: first, by introducing certain cultures into fresh populations and observing their mode of spread, and second, by titrating cultures under controlled conditions. A number of these tests and titrations will now be described.

The Spread of Friedländer-Like Bacillus Strains in Fresh Populations

Experiment 1.—This experiment was made to test the activity of an "endemic" strain. The technique and results were as follows:

The so-called "endemic" strain was obtained from Population 2 on Jan. 3, 1926. At that time the community was considered to be in an "endemic" phase, with respect to the Friedländer infection, for the previous epidemic had ended 2 weeks previously and, as later developed, no further outbreak took place for 8 weeks.

[1] The term "virulence," when used in an epidemiological sense, is defined according to the present state of knowledge, in the most general terms as "pathogenicity" of a microbe.

Consequently, a culture was taken on Jan. 3 from the heart's blood of a mouse in the population, found dead on that day. On Jan. 7, this culture, grown 18 hours in broth and diluted 100 times in salt solution, was given to 20 mice by instilling 2 drops into the nares of each. These 20 mice, designated Population 5, were then placed in a single standard cage and given the routine care. Each day two fresh immigrants were added and dead animals were removed and examined (1 a).

The results of this procedure are shown in Text-fig. 1. 2 days after inoculation, 1 mouse died; on the third day, 8 mice, on the fourth day, 3, and on the fifth day, 4 mice. On the sixth day, 2 mice died, one of which was an immigrant added Jan. 8; on the seventh day, 3 died, including an immigrant of Jan. 9; on the eighth day, 2 died, and on the ninth day, the last individual of the original 20 died. All dead mice were autopsied and found positive for the Friedländer bacillus. *B. enteritidis* (1 c) was likewise recovered 10 days after the community was established. Following the first series of deaths, the population census increased to 43 individuals, when a secondary outbreak occurred. In 4 days, 17 mice died and the number dropped to 34. The 14 animals showed typical Friedländer pneumonia and yielded the organisms in pure culture (Text-fig. 1). After the second outbreak, the number again rose to 46, when the third outbreak occurred. This time, 28 animals died in 7 days and the census fell to 32. Again the population count turned upward to 46, when the fourth outbreak ensued, fatal to 38 animals in 5 days. At once, deaths ceased abruptly; the population increased without interruption from 17 to 80, and the Friedländer infection disappeared.

This experiment demonstrated, first, that a culture of the Friedländer organisms removed from Population 2 during an inter-epidemic period was able to become established in a fresh community, to spread and give rise to typical epidemics. Most important, however, was the fact that these epidemics resembled closely the previous outbreaks in Populations 2 and 4. The comparison, shown in Table I, indicates that the primary and secondary outbreaks in Populations 2, 4, and 5 were alike in occurring at a census level of about 45, in lasting about 7 days, and in reducing the census to about 27 individuals. Finally, it should be noted that the Friedländer disease disappeared from both Populations 2 and 5 at about the same time. It is concluded, therefore, that the pathogenicity of the "endemic" strain of Friedländer was similar to that of the original "epidemic" strains.

Experiment 2 was made to test the activity of an "inter-epidemic" strain. The technique and results of the experiment were as follows:

On Nov. 5, a fresh population, designated No. 6 (Text-fig. 1), was established by assembling ten Rockefeller Institute mice in a standard cage and adding two individuals each day thereafter. On Dec. 29, with the population numbering 101

individuals, the two immigrants were given intranasally about 100 bacilli of the "inter-epidemic" Friedländer strain, No. 18. This procedure was repeated on the 2 following days with the immigrants, so that a total of six "carriers" were introduced into the fresh, unexposed community.[2] Following this, the daily addition of normal mice was resumed. The "inter-epidemic" strain, No. 18, was obtained from the nasal passages of a "healthy" Lathrop mouse of Population 1 on Dec. 21, midway between the third and fourth epidemic waves. This community had received a daily quota of two Lathrop mice instead of Institute mice since Oct. 6, 1926 (see page 922 for details).

The results of this experiment are shown in Text-fig. 1. The "experimental-carrier" immigrants died in 5, 35, 15, 15, 9, and 50 days respectively. Notwithstanding the relatively long survival time (page 919), the infection spread rapidly.

TABLE I

Character of B. friedlaenderi-Like Epidemic Waves, 1925–6

Population identification	Epidemic waves Chronological number	Population at onset of each epidemic	Population at end of each epidemic	Duration of each epidemic in days
No. 2	2	47	16	7
" 4	2	44	18	6
" 2	3	34	26	5
" 2	4	38	29	6
" 2	5	52	34	14
" 2	6	56	39	6
" 5	2	43	34	3
" 5	3	46	32	6
" 5	4	46	17	10
	Av. 45.1 a.d. 5.1		Av. 27.2 a.d. 7.1	Av. 7 a.d. 2.1

The number of daily deaths increased quite regularly to a peak of 13 on Jan. 12. By Jan. 19, 15 days in all, 88 mice had died and the number dropped to 46. Throughout the epidemic, the dead mice showed typical Friedländer bacillus lesions, and yielded the organisms either in pure culture, or in combination with *B. enteritidis*. Following this primary outbreak, the number rose in 22 days to 69, when a second explosive outbreak occurred. This epidemic reached its peak on the fifth day, with the death of ten mice, and lasted 13 days, with a total mortality of 85. Subsequently, the population count rose steadily from 10 to 94 and the infection disappeared permanently.

This experiment demonstrated that an "inter-epidemic" strain from the nasal passages of a clinically healthy individual possessed the

[2] Two tests for nasal carriers, Dec. 1 and 28, proved negative.

various potentialities of and was similar to the so-called "epidemic" strains. It spread rapidly throughout a fresh community, giving rise to epidemic outbreaks and disappearing at about the same time as the infections in Populations 2 and 5.

Experiment 3 was made to retest the findings of Experiment 2.

On Feb. 18, 1927, two groups of 100 Institute mice were placed in standard cages and designated Populations 9 and 10. They were then retained for observation for a period of 33 days, during which time they remained free of the Friedländer bacillus disease,[3] but suffered some loss through fighting. These losses were made up by the periodic addition of immigrants, so that the populations remained approximately at 100 individuals. On Mar. 23, 12 additional mice were given intranasally about 768 bacilli of the Friedländer strain, No. 18, used in the previous experiment. Six of these mice were then placed in Population 9 and 6 in Population 10. 4 days later the immigrants began to die and explosive outbreaks of great severity took place in both communities (Text-fig. 1). Following this, the infection assumed an endemic form and finally, after 2 to 3 months, disappeared completely. The daily addition of immigrants was commenced on April 8.

This experiment confirmed the findings of the previous test, showing that the "inter-epidemic" Strain 18 was similar to the "epidemic" strains in its ability to spread and give rise to severe epidemics and later, to disappear.

Virulence Titrations of the Friedländer-Like Organisms

Experiment 4.—Direct titrations of the Friedländer organisms were made to determine their virulence during various endemic and epidemic phases of the infection. In all, seven tests were carried out on a total of 15 strains, 3 taken from inter-epidemic periods, 2 from pre-epidemic, 8 from epidemic, and 2 from post-epidemic periods (Table II). In each instance an effort was made to compare strains taken from populations at the time of the greatest differences in severity of infection. Full details of these tests are given in another publication (1 a); it will suffice here to give the exact source of each strain and the results of the tests in terms of final 60 day mortality figures (Table III).

Titration 1.—Nov. 22, 1926. Two "inter-epidemic" 1926 strains, Nos. 1 and 4, and one "epidemic" 1925 strain, No. 6, were compared. Strains 1 and 4 were

[3] Two tests for the presence of nasal carriers proved negative.

obtained from Population 1. Strain 1 came from the heart's blood of a Lathrop mouse found dead Oct. 25, a date between the third and fourth epidemic outbreaks; Strain 4 from a similar Lathrop mouse, Nov. 17, between the fourth and fifth outbreaks. Strain 1 was obtained originally from a mouse in Population 4, dead at the peak of the second epidemic wave, Oct. 13, 1925 (1 b). Each strain was administered intranasally to 50 mice; the resulting mortalities were: Strain 1, 84 per cent; Strain 4, 86 per cent; Strain 6, 74 per cent.

Titration 2.—Dec. 16, 1926. One "inter-epidemic" strain, No. 1, and two "pre-epidemic" strains were compared. Strain 1 has been described; Strains 11 and 12 came from Population 5, 1 and 7 days before a severe outbreak. Strain 11 came from the nasal passages of a "healthy" mouse (No. 7–32) which had been in the community 12 days, but died the day following the taking of the culture (Dec. 9). Strain 12 came from the nasal passages of "healthy" Mouse 4–24,

TABLE II

Source and Number of B. friedlaenderi-Like Strains Used in the Virulence Titrations

Population identification	Period of infection at which the strains were obtained				
	Number of strains				
	Inter-epidemic period	Pre-epidemic period	Epidemic period	Post-epidemic period	Total number
No. 1	3		1		4
" 4			1		1
" 5		2	3		5
" 6			3	1	4
" 10				1	1
Total number.....	3	2	8	2	15

which had been in the community 95 days and a carrier for at least 17 days, but which died 9 days after the taking of the culture (Dec. 3). Each strain was instilled intranasally to 40 mice; the ensuing mortalities were: Strain 1, 70 per cent; Strain 11, 85 per cent; Strain 12, 55 per cent.

Titration 3.—Dec. 20, 1926. One "inter-epidemic" strain, No. 1, and two "epidemic" strains, Nos. 13 and 15, were compared. Strain 1 has been described; Strains 13 and 15 came from Population 5, at the peak of the December outbreak. Strain 13 was procured on Dec. 14 from the nasal passages of Mouse 7–30, which had been in the community 44 days and died 1 day after the culture was obtained. Strain 15 was taken, on Dec. 14, from the nasal passages of Mouse 8–33, a resident of the community 12 days and dead 12 days later. Each strain was administered intranasally to 50 mice; the final mortalities were: Strain 1, 90 per cent; Strain 13, 80 per cent; Strain 15, 72 per cent.

TABLE III

Virulence of B. friedlaenderi-Like Organisms Obtained from Populations during Various Pre-, Epidemic, and Post-Epidemic Periods of the Disease

Date of test	Titration number	Strain number	Population source	Individual source	Date obtained	Phase of disease	Number of mice tested	Total mortality
11/22/26	1	1	1	Lathrop mouse. Autopsy—blood culture	10/25/26	Inter-epidemic*	50	84
"		4	1	" " " " "	11/17/26	" *	50	86
"		6	4	R. I. " " " "	10/13/25	Epidemic peak	50	74
12/16/26	2	1	1	Lathrop mouse. " " "			40	70
"		11	5	R. I. mouse—Carrier No. 7–32. Nasal passage (1)	12/ 9/26	Pre-epidemic (24 h.)	40	185
"		12	5	R. I. mouse—Carrier No. 7–24. Nasal passage (2)	12/ 3/26	" (7 days)	40	55
12/20/26	3	1	1	Lathrop mouse. Autopsy—blood culture			50	90
"		13	5	R. I. mouse—Carrier No. 7–30. Nasal passage (3)	12/14/26	Epidemic peak	50	80
"		15	5	R. I. mouse—Carrier No. 8–33. Nasal passage (4)	"	" "	50	72
1/ 7/27	4	1	1	Lathrop mouse. Autopsy—blood culture			50	79
"		16	1	" " " lung "	12/30/26	" "	50	72
"		17	5	R. I. mouse. " " "	"	" "	50	92
1/19/27	5	18	1	Lathrop mouse—Carrier No. 33. Nasal passage (5)	12/21/26	Inter-epidemic**	100	70
"		19	6	R. I. mouse. Autopsy—lung culture	1/12/27	Epidemic peak	100	65
2/28/27	6	19	6	" " " " " "		" "	50	82
"		32	6	" " " " " "	2/17/27	" "	50	70
"		33	6	" " " " " "	"	" "	50	76
"		34	6	R. I. mouse. Carrier No. 10–9. Nasal passage (6)	2/23/27	Post-epidemic	50	82
6/ 2/27	7	35	10	R. I. mouse—Carrier. Nasal passage (7)	6/ 1/27	"	50	80

* Designated "pre-epidemic" in earlier report (1 a).

** Designated "epidemic" in earlier report (1 a).

(1) Died 1 day later.

(2) Date of death not known.

(3) Died 1 day later, 12/15/26 (error in description in 1 a).

(4) Died 12 days later, 12/26/26 (" " " " ").

(5) This strain was used to infect Populations 6, 9, 10.

(6) Died 5 days later, 2/28/27.

(7) Designated "Str. 50" and "Str. 35" in earlier report (1 a).

Titration 4.—Jan. 7, 1927. One "inter-epidemic" strain, No. 1, and two "epidemic" strains, Nos. 16 and 17, were compared. Strain 16 came from the lung of a Lathrop mouse in Population 1, found dead Dec. 30, at the peak of an epidemic outbreak; Strain 17 was obtained from the lung of Mouse 7–35 of Population 5, found dead Dec. 30, during the height of a prolonged epidemic. Mouse 7–35 had been in the population 9 days. Strain 1 was administered intranasally to 100 mice, Strains 16 and 17 similarly to 50 mice each. The resulting mortalities were: Strain 1, 79 per cent; Strain 16, 72 per cent; Strain 17, 92 per cent.

Titration 5.—Jan. 19, 1927. One "inter-epidemic" carrier strain, No. 18, and one "epidemic" strain were compared. Strain 18 was obtained from the nasal passages of a clinically healthy Lathrop mouse in Population 1, Dec. 21, midway between the fourth and fifth epidemic waves. Strain 19 came from the lung of a mouse in Population 6, found dead Jan. 12, 1927, at the peak of the primary epidemic outbreak. Each strain was administered intranasally to 100 mice; the resulting mortalities were: Strain 18, 70 per cent; Strain, 19, 65 per cent.

Titration 6.—Feb. 28, 1927. Three "epidemic" strains, Nos. 19, 32, and 33, and one "post-epidemic" strain, No. 34, of the same population, No. 6, were compared. Strain 19 has been described; Strains 32 and 33 were obtained from the lungs of 2 mice, Nos. 10–2 and 2–4, found dead Feb. 17, at the peak of the second epidemic wave. Mouse 10–2 had been in the community 83 days, and Mouse 2–4 71 days. Strain 34 came from the nasal passages of clinically healthy Mouse 9–10, on Feb. 23, as the epidemic was subsiding. This mouse, added to the community 10 days before the culture was obtained, died 5 days later. Each strain was given intranasally to 50 mice; the final mortalities were: Strain 19, 82 per cent; Strain 32, 70 per cent; Strain 33, 76 per cent; Strain 34, 82 per cent.

Titration 7.—June 2, 1927. One "post-epidemic" strain was tested. Strain 35 was taken from a mouse in Population 10, 2 months after the previous epidemic and just before the disease disappeared permanently. The present strain, No. 35, was obtained from the nasal passages of a clinically healthy mouse which had survived the epidemic. 6 days later, a second nasal passage culture failed to yield the Friedländer organisms. This strain was given intransally to 50 mice with a resulting mortality of 80 per cent.

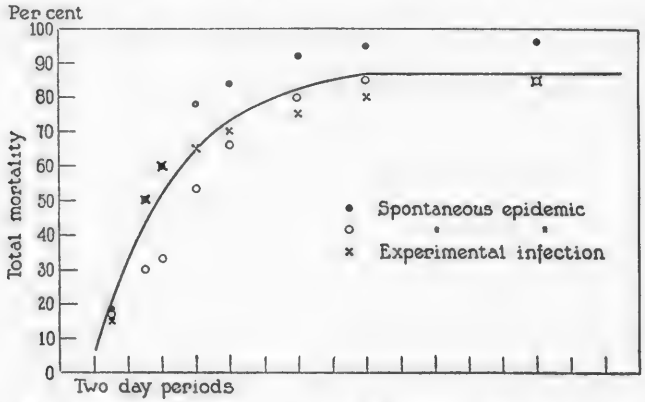
The results of these titrations demonstrated that the virulence of these 15 strains was essentially the same and indicated: 1) that the 1925 to 1926 Friedländer strains were similar to the 1926 to 1927 strains, 2) that strains from Population 1, in which frequent epidemics occurred in 1926 to 1927, were of the same disease-producing power as strains from Population 5, in which during the same period the disease was less severe, and finally 3) that the pre-epidemic, epidemic, post-epidemic, and inter-epidemic strains were of similar disease-producing power or virulence. It was concluded, therefore, that the

microbic virulence factor remained constant during the various endemic and epidemic phases of the Friedländer bacillus disease.

Host-Resistance to the Infection

The behavior of the host-resistance factor in the various populations during epidemic phases was analyzed in the following manner.

Host-Resistance during Pre-Epidemic and Epidemic Periods.—In previously unexposed communities, differences in the individual resistances of population con-



TEXT-FIG. 2. Comparison of mortality in "spontaneous" epidemics and experimental Friedländer bacillus infection. Two "spontaneous" epidemics in Populations 9 and 10, April, 1927 (Text-fig. 1) are compared with average mortality figures of standard titrations (1 b, Text-fig. 1). The figures are plotted in terms of total cumulative mortality.

stituents appeared to determine the form of the primary epidemic wave. Thus, a comparison of mortality curves of primary epidemics (Text-fig. 1) with mortality curves following the experimental inoculation of groups of mice with equal numbers of a single strain of Friedländer bacilli (Text-fig. 1) (1 b) showed close agreement in amount and distribution of mortality (Text-fig. 2). The mortality rate in the case of mice receiving experimental inoculation under conditions of known constant virulence and dosage was definitely attributable to differences in individual resistance. Hence in the case of similar mice undergoing a primary epidemic in which large doses of a foreign microorganism were available, the mortality rate, or form of the epidemic curve was likewise considered due to differences in individual resistances of the population constituents. A similar comparison has been made previously with "spontaneous" epidemic curves and experimental titration curves

in mouse *B. aertrycke* infection (Text-fig. 4) (1 d). *In communities infected* with the Friedländer bacilli, epidemic outbreaks appeared to be caused by a progressive decrease in the host resistance factor to a critical point. Under the given conditions each population received two mice daily from the Institute stock, whose average resistance was known to be constant (1 e). Furthermore, the average resistance of these fresh immigrants was known to be less than that of the older residents (page 920). Hence the daily immigrants lowered the average resistance of the total population at a relatively constant rate. That this decrease in population resistance was responsible for the outbreaks is indicated by the occurrence of epidemics at definite population levels, their duration for a definite number of days, and reduction of population numbers to a definite level. For example, in 1925 and 1926, epidemics arose in Populations 2, 4, and 5 at an average census level of 45.1 days, lasted 7 days, and reduced the populations to 27 individuals (Table I); in 1926, primary epidemics occurred in Populations 1, 2, and 5 at census levels of 65 to 82, lasted 23 to 35 days, and reduced the numbers to 20 to 28 individuals; and secondary outbreaks (2, 3, 4) in Population 1 were similar in these respects to those of 1925 (Table V). This similarity in numbers of population at the onset of epidemics indicates that the daily immigration of two fresh mice decreased the average resistance of the total population from a high to a critically low level;[4] the similarity in both the duration of epidemics and the census levels at the end of outbreaks is probably due to the deaths of susceptible individuals in numbers sufficient to raise the average resistance of the surviving population to a critically high level. That susceptible immigrants were actually responsible for the spread of infection and onset of epidemics is probable from the records of survival time. The figures of survival time, that is, of the average duration of life of two immigrants of a given day (Text-fig. 1), decreased prior to epidemic outbreaks and increased sharply before the peak mortality of each epidemic was reached. For example, preceding the Aug., 1925, Pasteurella epidemic in Population 2 (1 a), the average survival time of immigrants decreased steadily from 30 to 7 days, July 18 to Aug. 10, while during the 4 pre-epidemic and 4 epidemic days, Aug. 11 to Aug. 19, it underwent irregular alterations but increased to an average of 18 days. Prior to the Sept., 1925, Friedländer epidemic in Population 2 (1 a), the survival time during the period Aug. 29 to Sept. 5 decreased steadily from 27 to 5 days, while 3 days before the outbreak it commenced to rise, and during the epidemic maintained an average level of 25 days. Prior to the Friedländer epidemic Oct. 2, 1925, in Population 2, the survival time of immigrants dropped steadily from 26 to 5 days, (between Sept. 18–30) but 24 hours before the onset of the epidemic it rose sharply, and during the wave maintained a relatively high average figure. In the case of prolonged epidemics with high plateau form of mortality, the survival time decreased prior to the outbreak, rose sharply imme-

[4] Topley and Greenwood have pointed out a direct relation between immigration rate of susceptibles and frequency and severity of epidemics (*J. Hyg.*, 1925, 24, 45).

diately preceding or during the early phases of the waves, fell again to a low level at the time of steady high mortality, and finally rose again shortly before the terminal drop in number of deaths. This relationship between survival time and epidemic phase was best shown in such epidemics as that in Population 2, Aug., 1926 (Text-fig. 1). These fluctuations in survival time were paralleled by fluctuations in carrier rate (page 925). Consequently, it is probable that in the given communities infected with microorganisms of constant virulence, fresh immigrants decreased population resistance to a critical level, provided fertile soil for the multiplication of the microbes, and thus became the incitants and first victims of the epidemic.[5]

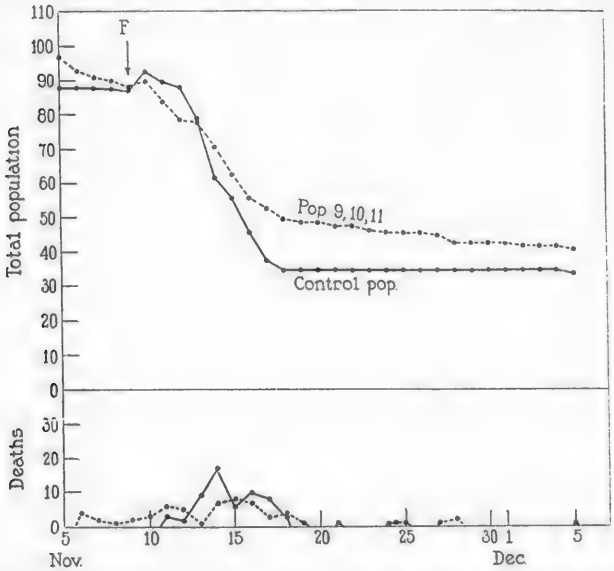
Host-Resistance during the Post-Epidemic Phase.—The survivors of a Friedländer epidemic proved to be the relatively resistant individuals. Thus, for example, the survivors of a "spontaneous" epidemic were found to correspond in relative numbers and behavior to the survivors of an experimentally inoculated series of mice (Text-fig. 2), indicating that both survivor groups were selected, resistant survivors, not specifically immunized individuals. Again, survivors tended not to be "carriers" (page 925), nor to succumb during the early phase of a succeeding epidemic. Large numbers of survivors in a community decreased the number and severity of epidemics (Text-fig. 1). Again, communities of survivors unaccompanied by susceptible immigrants tended to become free of the infection. An example of this fact was the event which took place in Populations 3, 4, and 1, Nov., 1925 (1 a) (Text-fig. 1). Following the Friedländer outbreaks of Sept., 1925, immigrations to Populations 3 and 4 were discontinued and the survivors were added to the uninfected Population 1 at the rate of two per day in place of the usual immigrants. On Nov. 23, the regular mode of immigration was resumed. No spread of the Friedländer disease to Population 1 occurred; neither immigrating survivors nor residents gave evidence of infection. Thus it is inferred that survivors of a given infection are relatively more resistant than a random unexposed group (2–3).

Experiment 4.—A direct measurement of the resistance of survivors was made in the following manner.

Populations 9 and 10 (Text-fig. 1), free of the Friedländer infection in July, 1927, showed populations on Oct. 5 of 103 and 104 respectively. At this time, five immigrant mice, each just inoculated intranasally with about 425 bacilli of the Friedländer Strain 18 were substituted in each population for the usual daily quota of two immigrants. At the same time, a fresh community, consisting of 110 standard Institute mice and designated Population 11, was treated in a similar

[5] Topley (2) and Amoss (3) have pointed out that in experimental mouse typhoid epidemics the recent immigrants die sooner than the older inhabitants. Webster (1 g) has shown that survivors of experimental mouse typhoid infection are more resistant than non-exposed animals.

manner. Further additions were discontinued. Within 10 days, the infected immigrants were dead and typical explosive Friedländer epidemics raged in each community. On Oct. 27, Population 9 was reduced to 45 survivors, Population 10 to 36, and Population 11 to 43, indicating that each community contained about the same amount of susceptible and resistant material. On Oct. 27, 5 mice inoculated intranasally with 400 to 500 bacilli of Strain 18 were added to each community. Subsequently, few individuals died and repeated tests for nasal carriers became negative on Nov. 4. The second test was made Nov. 9 by combining the



TEXT-FIG. 3. Relative resistance of "survivors" and "normals" to Friedländer-like bacillus infection.

survivors of Populations 9, 10, and 11 in one cage and adding to them 5 mice inoculated intranasally with 400 to 500 Friedländer bacilli of Strain 18. At the same time a control population of 88 fresh Institute mice received 5 similarly inoculated mice. The results of this test are shown in Text-fig. 3. Mice in the control community died more rapidly than those in the "survivor" community, indicating that the immediate survivors of a Friedländer epidemic were slightly but definitely more resistant than mice not exposed to the infection.

The facts presented suggest that host resistance plays the determining rôle in the spread of the Friedländer infection. Thus, in previously

uninfected communities, the average level of resistance to a fixed microbic virulence appeared to determine the amount and severity of the infection, and individual differences in resistance to determine the time distribution of mortality; whereas in already infected communities, a lowering of the amount of host resistance through immigration of mice with relatively low average resistance seemed to act as the underlying cause of epidemic outbreaks; and finally, the resistance of survivors of an epidemic was found to be greater than that of unexposed individuals and to be more general than specific in nature.

An experiment was made to determine the effect of varying host resistance on the spread of the Friedländer infection. The test was carried out in the following manner.

Experiment 5.—A change in the amount of host resistance in Population 1 was effected by substituting for the daily immigration of two standard Institute mice (1 a), two Lathrop mice.

TABLE IV

Relative Susceptibility of Lathrop and Rockefeller Institute Mice to B. aertrycke (M. T. 2) Mouse Typhoid

Race	1923			1924												1925								
	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.
Rockefeller Institute	78	74	66	78	90	78	58	86	52	40	64	80	56	48	88	38	56	66	94	86	92	81	84	86
Lathrop	66		64	76	82	80	78	91	80	78	76	94	84	70	89	84	66	94	96	87	86	91	88	100

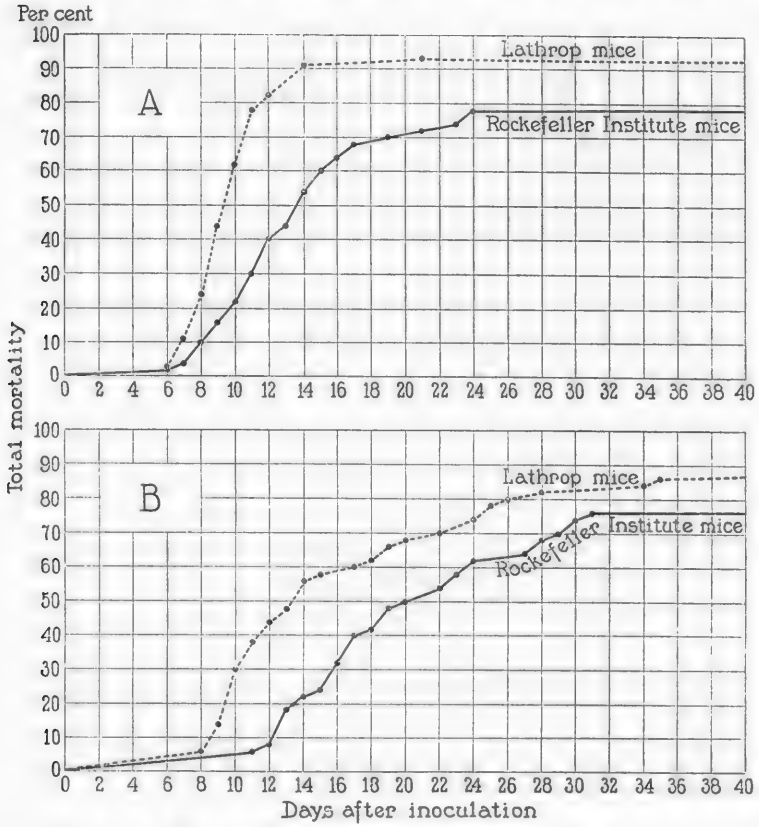
Total number used: Rockefeller Institute mice..... 1230
Lathrop mice..... 1306

The Lathrop mice have been subjected previously to repeated tests and found to be less resistant to infection than the Institute strain. These preliminary tests were made as follows:

Test 1.—Pritchett (1 e) in a series of 23 monthly tests on a total of 2536 mice has shown the Lathrop strain to be less resistant than the Rockefeller Institute strain to *B. aertrycke* mouse typhoid. For each titration, 50 animals of each strain, raised under similar conditions, without previous exposure to the infection, were given *per os* a uniform dose of the bacteria. For the first 4 tests, it was necessary to use old breeders of the Lathrop strain; subsequently, standard Lathrops 12 to 14 weeks old, weighing 16–18 gm., were compared with similar standard Institute mice. The results, summarized in Table IV, showed that under the given set of

conditions, 18 of 19 properly controlled groups of Lathrop mice and 19 of 23 total groups showed a higher mortality than the Institute mice.

Test 2.—More recent tests have been made with *B. enteritidis* mouse typhoid bacilli. On two occasions, Dec., 1927, and Mar., 1928, 50 animals of each strain



TEXT-FIG. 4. Relative resistance of Lathrop and Rockefeller Institute mice to *B. enteritidis* infection.

were given *per os* the usual dose (1 e) of strains of *B. enteritidis* obtained from Populations 5 and 1. The results shown in Text-fig. 4 indicated that the Lathrop mice were less resistant than the Institute mice to these organisms.

The substitution of Lathrop immigrants for those of the Institute strain migrants in Population 1 was made at a time when the three populations were behaving in a similar manner, thus permitting the use of one community for the test and two communities for control. The substitution of Lathrop mice in Community 1 was commenced on Oct. 6 and continued to Jan. 7, 1927. The results of this substitution were striking. In contrast to the events in control Population 2, in which the infection disappeared early in November, and control Population 5, wherein, after one further atypical outbreak, the infection disappeared in Jan., 1927, the events in test Population 1 were a rapid succession of five explosive epidemics, similar to those observed in the 1925 to '26 outbreak (Tables I, V). Furthermore, after the addition of Lathrop mice was discontinued on Jan. 7 and Institute immigrants were substituted, the Friedländer infection disappeared as it had done in control Populations 2 and 5, and the population numbers in all communities became similar.

TABLE V

Character of Epidemics of Friedländer-Like Bacillus Infection, 1926–27

Population identification	Epidemic waves Chronological number	Population at onset of each epidemic	Population at end of each epidemic	Duration of each epidemic in days	Population identification	Epidemic waves Chronological number	Population at onset of each epidemic	Population at end of each epidemic	Duration of each epidemic in days
No. 1	1	81	24	28	No. 1	2	40	27	9
" 2	1	82	28	35	" 1	3	36	21	10
" 5	1	65+	20	23+	" 1	4	35	25	10
" 2	2	41	26	9	" 1	5	56	41	11
" 5	2	69	54	20	" 1	6	60	40	11
" 5	3	82	25	26	" 1	7	53	45	11

The result of this experiment, charted in Text-fig. 1, shows how closely events in Population 1, up to the time of adding the Lathrop mice, paralleled those in the control Populations 2 and 5; how thereafter the infection in Population 1 assumed a far more serious nature than that in Communities 2 and 5; and finally, how, when the addition of Lathrops was discontinued in Population 1 and the Institute breed substituted, the character of the infection changed again to that prevailing in the control communities and the daily census figures again became very much alike. The observations, made under conditions so well controlled, were also accompanied by virulence titrations of Friedländer cultures, which showed that cultures from the test Lathrop

community (Population 1) and the control community (Population 5) were of the same pathogenicity (page 914). Consequently, it is difficult to escape the conclusion that the factor actually increasing the severity of infection in Population 1 was the substitution of the more susceptible Lathrop for the Institute mice, and that the subsequent addition of the latter brought about the fall of the infection to the level of that in the other communities. It is concluded, therefore, that under the conditions of the experiment, the decrease in population resistance brought about typical epidemics of the Friedländer infection, and the subsequent increase in population resistance brought about a diminution in its severity and spread.

Effect of the Dosage of Friedländer Organisms

The third epidemic factor investigated was microbic dosage, the numbers of Friedländer organisms available to a community during various phases of the infection. Preliminary searches for the bacilli in the feces and cage bedding were fruitless and indeed, tests for viability of Friedländer cultures mixed intentionally with bedding were negative after 72 hours. Further tests showed that Friedländer organisms were harbored in the nasal passages of certain individuals and these "nasal" carriers were capable of spreading the infection (page 911). Consequently, serial determinations of the nasal carrier rate in the several populations were made during the 1926–1927 period of the Friedländer infection.

The tests were carried out by culturing the nasal passages of each mouse in a given community. The animal was held firmly and 1 to 2 drops of salt solution were placed in and about the external nares. The head was directed in such a manner as to insure rapid inspiration and expiration of the fluid until it became slightly cloudy. This fluid was then spread over the surface of a plain agar plate; after 48 hours' incubation, the characteristic colonies were selected and identified in agglutinating antiserum mixtures (1 b).

Six tests were made in Population 2, Oct. 19, 1926 to Jan. 18, 1927, and 12 tests in Population 5, Nov. 16, 1926 to Jan. 29, 1927. In all, 21 carriers were found. Of these, two became negative for the Friedländer organisms and survived at least 60 days; the remainder died of the infection in 5 $\pm$ 3.5 days. The term during which the carriers had resided in the population prior to the test varied from 6 months to 2 days. Eleven tests were made in Population 1 during various phases of the Friedländer infection, Oct. 21, 1926, to Mar. 10, 1927. The first test, Oct.

21, 1926, following the third epidemic wave, showed 12.5 per cent carriers; the second test, Oct. 28, preceding the fourth outbreak, 27 per cent; after the fourth outbreak, Nov. 12 and 18, the rates were 10 per cent and 5.7 per cent. At the height of the fifth epidemic, Dec. 7, 20 per cent were carriers; before, during, and after the sixth wave, Dec. 21, 29, and Jan. 8, the rates were 5.5 per cent, 7 per cent, and 2 per cent respectively. At the peak of the seventh wave, Feb. 1, 13 per cent were carriers; four days later, no carriers were found. One further test, Mar. 10, was likewise negative.

The results of the carrier tests showed that "healthy" chronic carriers of the Friedländer organisms were infrequent, and that the microbes tended either to kill the host or to disappear. The tests likewise indicated that the dosage factor, as determined by the number of carriers, increased during the pre-epidemic and epidemic periods to a maximum near the epidemic peak, and subsequently decreased during the post-epidemic and inter-epidemic periods.

DISCUSSION

The studies in this and the preceding paper (1 a) furnish direct experimental evidence on the mode of spread of Pasteurella, Friedländer, and *B. enteritidis* (1 c) infections of mice. This evidence relates both to the behavior of communities previously unexposed to these infections and to communities in which the specific infection was already present. Concerning the primary response of unexposed communities, it was shown that three general results occurred, depending on the pathogenicity of the infecting microbe,—first, an epidemic reaction, involving explosive outbreaks of disease and subsequent disappearance of the specific agent (*P. avicida* and *B. friedlaenderi*); second, an endemic reaction involving sporadic endemic disease and permanent establishment of the agent in the community (*B. enteritidis*, (1 c)); and third, no reaction, leading to no disease, and no establishment of the agent (*P. lepiseptica*). These varieties of response under controlled conditions were proved to be due to qualities in the specific bacteria,—the epidemic type of reaction being caused by the virulent non-vegetative pathogens (*P. avicida* and *B. friedlaenderi*), the endemic type by the less virulent, highly vegetative forms (*B. enteritidis*), and the third by the least virulent, least vegetative forms (*P. lepiseptica*). Similar differences of response observed in previous studies of Pas-

teurella infections of rabbits (1 d) and chickens (1 f) were found likewise to be associated with the disease-producing power of the specific bacteria. Hence, it is concluded that in the diseases studied, the primary response of unexposed communities of given average resistance is determined by the inherent pathogenicity of the specific microbes.

In the case of communities already infected, the inherent pathogenicity of the specific microbes proved to be unchanging and the course of the infection to be controlled by the host-resistance variable. Thus, the virulences of *P. avicida* and *B. friedlaenderi* were constant whenever tested, strains obtained from populations during pre-epidemic, epidemic, post- and inter-epidemic periods behaving uniformly when introduced into fresh populations, or when titrated under controlled conditions. The host-resistance factor, on the other hand, appeared to vary in a definite manner according to the prevalence of the infection. Thus, prior to epidemic outbreaks, the survival time of immigrants declined progressively and the number of carriers and available bacteria (dosage) increased. Again, the actual outbreaks tended to occur at definite population levels and were most fatal to recent immigrants. And finally, the epidemics terminated at relatively definite population levels and in conjunction with a decline in dosage and increase in the survival time of immigrants. These observations indicate that the reduction of population resistance by the addition of fresh immigrants of definite and relatively low average resistance was responsible for the characteristic outbreaks. Most significant in this connection, however, were the experiments in which changes in population resistance brought about by substituting immigrants of different racial or strain resistance determined the severity of the disease. Thus, the substitution of relatively susceptible Lathrop strain immigrants for resistant Institute ones brought about an increase in the severity and number of epidemics, while the reverse substitution of resistant for susceptible immigrants led to the opposite condition of infrequent epidemics. The same sort of substitution, carried on over a 2 year period in the case of *B. enteritidis* infection (1 c) gave comparable results. Hence it is concluded that under the conditions described, the amount and severity of infection were controlled primarily by the host-resistance variable.

The third conclusion applicable to the behavior of both the previously unexposed and the infected communities is that the form of the epidemic curve was determined by differences in the resistance of individuals in the community. This conception is derived from a comparison of mortality rates of "spontaneous" epidemics on the one hand and of controlled experimental inoculations of groups of mice on the other (page 918). Such comparisons have given similar results in three infections and indicate that the epidemic curve in populations in which uniform exposure to a microorganism of stabile virulence takes place within a relatively short time expresses differences in individual resistance to the infecting organism.

A final consideration of the epidemiology of these infections is reserved for the last paper (1 c).

SUMMARY

1. In September, 1925, explosive and highly fatal epidemics of a Friedländer-like bacillus infection arose in three of four experimentally controlled mouse populations. In the populations to which daily additions were made, characteristic secondary waves ensued. These waves appeared at definite population levels, lasted a definite number of days, and reduced the population to a definite low census. Subsequently, endemic periods intervened, followed by the disappearance of the disease during March, 1926.

2. In August, 1926, renewed epidemics of the infection arose in all four populations. These outbreaks resembled one another but were more enduring than the preceding ones. Moreover, fewer secondary waves followed, and the disease disappeared in February, 1927.

3. The virulence or disease-producing power of the Friedländer-like organisms, as determined by the ability to spread and induce typical epidemics among populations of previously unexposed mice, and by direct inoculation titrations remained constant during pre-epidemic, epidemic, post-, and inter-epidemic phases of the infection.

4. The Friedländer-like organisms were not recovered in cultures from bedding, food, or feces, but they were obtained from the nasal passages. Mice harboring the organisms usually died within a few days of the test; a few, however, survived and ceased to be carriers. The carrier rate in a given community was found to increase prior to

epidemic outbreaks and to decrease shortly before the time of peak mortality.

5. The substitution of relatively susceptible for "standard" immigrants in a population was followed by an increase in severity and spread of the disease, whereas a substitution of "standard" for resistant immigrants was followed by a fall in severity and extent of disease.

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THE RÔLE OF MICROBIC VIRULENCE, DOSAGE, AND HOST RESISTANCE IN DETERMINING THE SPREAD OF BACTERIAL INFECTIONS AMONG MICE

III. B. ENTERITIDIS INFECTION

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(Received for publication, July 26, 1930)

The experimental epidemiological investigations described in the two preceding papers (1 a, b) were concluded by a similar study of *B. enteritidis* typhoid. The presence of this disease in the several test populations was utilized to collect data on its mode of spread and its behavior relative to the bacteriophage and bacterial variation phenomena. Furthermore, tests were made, during characteristic phases of the infection, of the variability of the epidemic factors and of the effect of experimentally altering the host-resistance factor. These studies are described in the present paper.

Technique

The populations of mice employed in this work were the same as those used previously (1 a, b). It may be recalled that several communities were established and maintained in an essentially similar manner, and that each, unless otherwise noted, received a daily increment of two Rockefeller Institute mice. The history of each population may be summarized as follows: Populations 1, 2, 3, and 4, established and exposed to Pasteurellae June, 1925, became free of these infections within a few weeks. In Sept., 1925, all save Population 1 experienced outbreaks of a Friedländer-like bacillus respiratory disease. Subsequently, Population 2 suffered repeated epidemics until Mar., 1926, while Populations 3 and 4 were eliminated in Nov., 1925. A new population, No. 5, established Jan., 1926, was infected experimentally with the Friedländer bacillus. During the spring and summer of 1926, all communities became free of the infection; during Aug., 1926, however, all experienced renewed outbreaks which persisted in a mild form until Mar., 1927. Population 6, established Nov., 1926, was infected experimentally with the Friedländer bacillus and became free in Mar., 1927. Populations 9 and 10, established Feb., 1927 and similarly infected with the Friedländer bacillus, continued to harbor the infection until July, 1927. Subsequently, Nov., 1927, these two communities were eliminated.

General Observations on the Spread of B. enteritidis Infection

B. enteritidis infection was noted relatively early in the above described communities, namely, 48, 7, 77, 43, 10, 64, 35, and 41 days respectively, after the date of their establishment. In each instance, the source of infection was probably a "healthy" carrier among the original population or the daily additions (1 a).

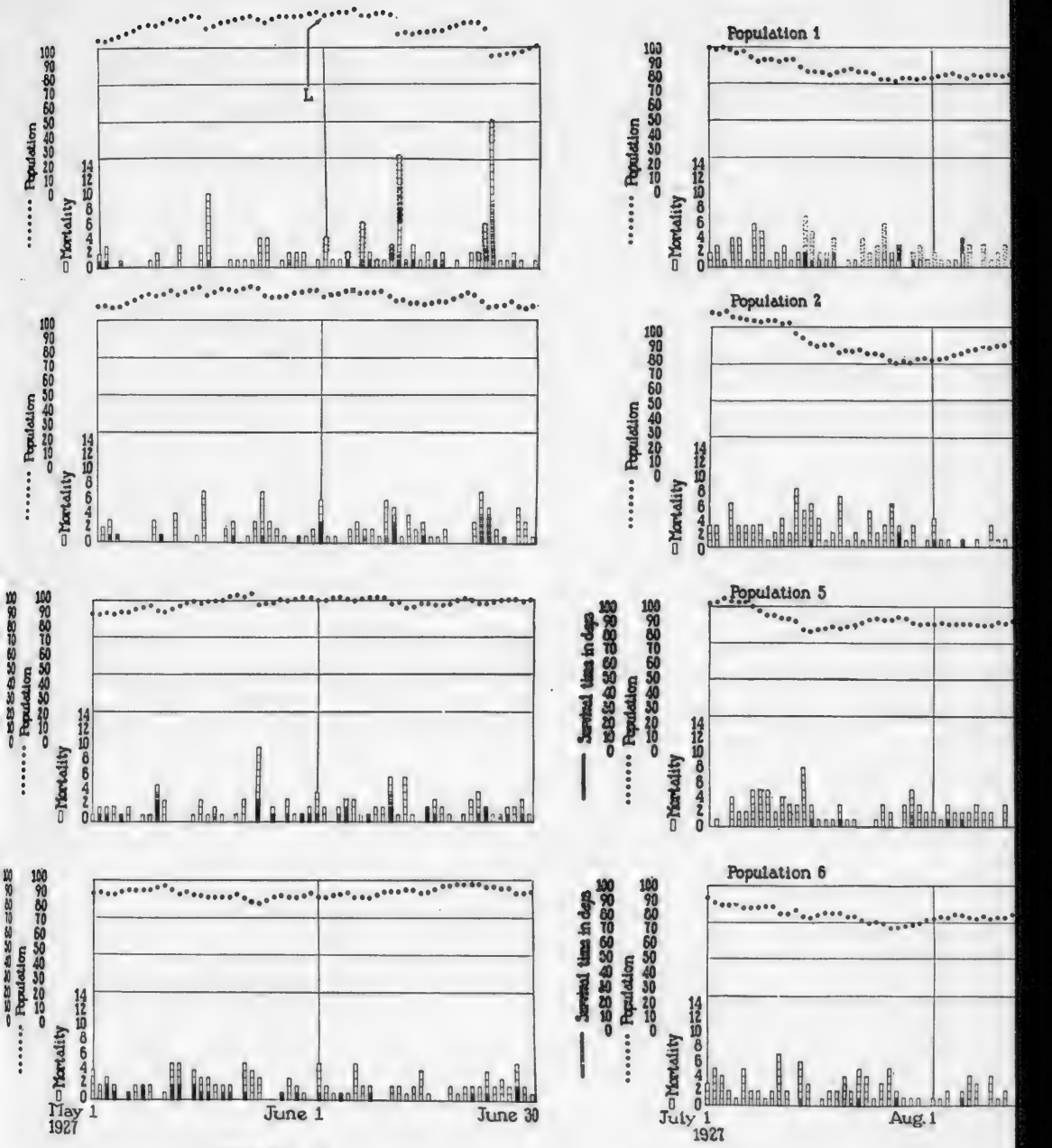
Observations on the spread of *B. enteritidis* typhoid in mouse populations were confined to periods in which the infection was prevailing in uncomplicated form. These periods were from Jan. to Aug. 1925, and Jan. 1928 to June 1929 in Population 1, Apr. to Aug. 1925 and Jan. 1927 to June 1929 in Population 2, May to Aug. 1925 and Apr. 1927 to June 1929 in Population 5, and May 1927 to June 1929 in Population 6.

TABLE I

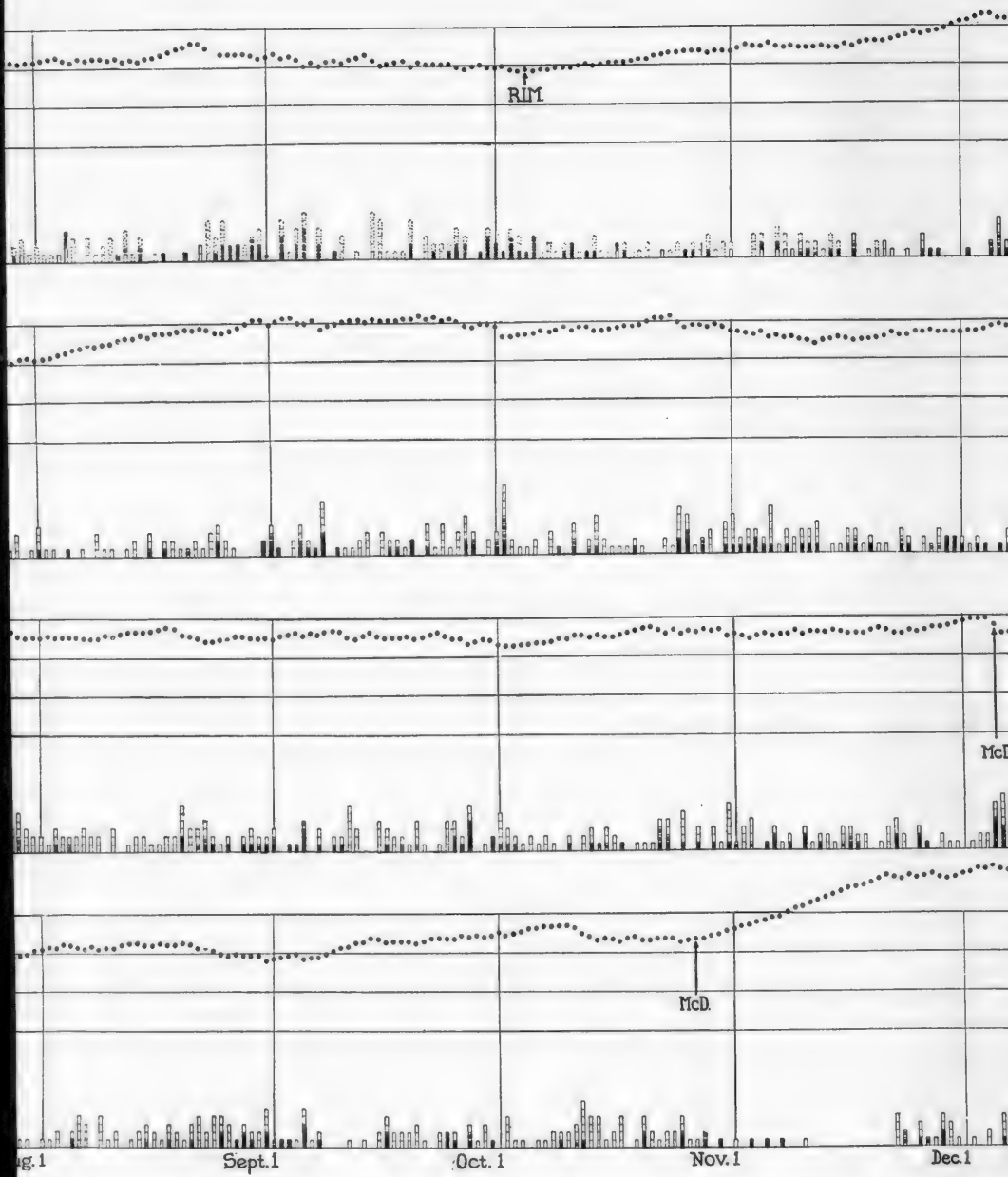
Total Mortality per Day in Population Infected with B. enteritidis

Population No.	Period	No. days	Initial population	Final population	Immigrants	Total deaths	Deaths per day
1	1/1/26–8/1/26	212	131	77	416	470	2.22
1	4/1/27–5/30/27	61	106	123	122	105	1.72
1	1/1/28–1/1/29	365	96	108	730	718	1.97
2	4/1/26–8/1/26	122	82	73	238	247	2.00
2	1/1/27–4/1/28	447	87	101	894	880	1.97
5	5/1/26–8/1/26	92	81	67	178	192	2.09
5	4/1/27–12/1/27	244	115	97	488	506	2.07
6	5/1/27–10/26/27	179	92	83	358	367	2.05

In general, the *B. enteritidis* infection consisted of periods of low mortality interspersed with epidemiform outbreaks. The occurrences of low mortality were similar in all populations. At these times, the census level averaged about 95 individuals and the mortality rate 2 individuals per day (Table I). Occasionally the census level and mortality rate continued constant for several days; more often, however, they fluctuated in a characteristic wave-like manner at intervals of about 9 days (Text-fig. 1). The periods of epidemiform mortality were of two sorts according to their duration (Text-fig. 1; Table II). One sort consisted of short periods of high mortality associated invariably with some sudden change in environment. In these instances, a return of the environment to "standard" conditions was accompanied by a cessation of deaths and an increase of the census to its usual level (Text-fig. 1). The second form of disturbance consisted of protracted periods of high mortality during the hot months of July and August (Text-fig. 1). Both types of environmental disturbance affected most severely the

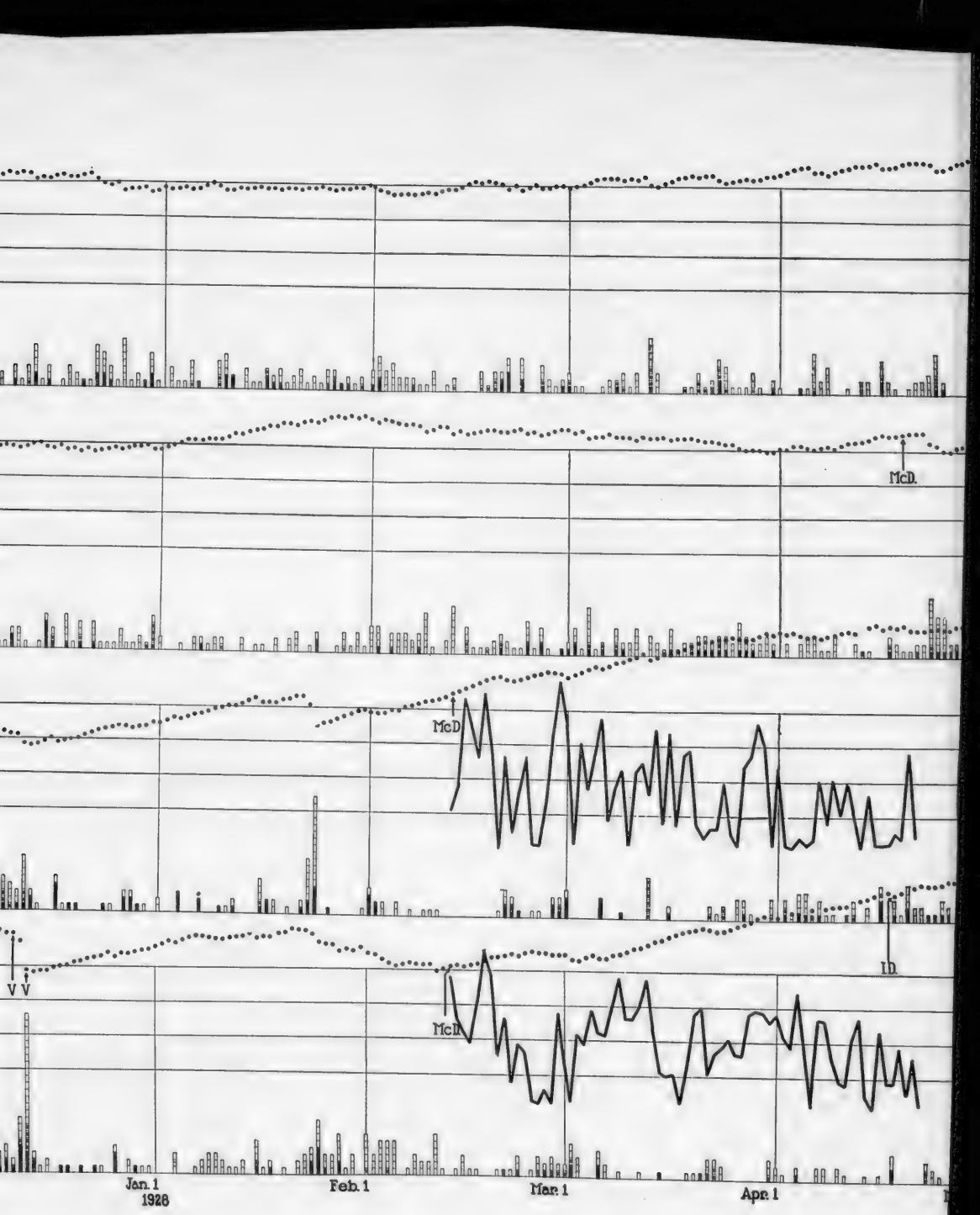

Population 1
Population 2
Population 5
Population 6
L
Population
Mortality
Survival time in days
May 1
1927
June 1
June 30
July 1
1927
Aug. 1

TEXT-FIG. 1*a*



TEXT-FIG.

TEXT-FIG. 1, *a*, *b*, and *c*. Spread of *B. ent*



tidis infection in mouse populations.

Survival time in days
Population
Mortality
May 1
June 1
June 30
1 mouse dead, no autopsy
1 " " autopsy findings negative
1 " " " Bact friedlander
1 " " " Bentertidis
1 Lathrop mouse
No mice added

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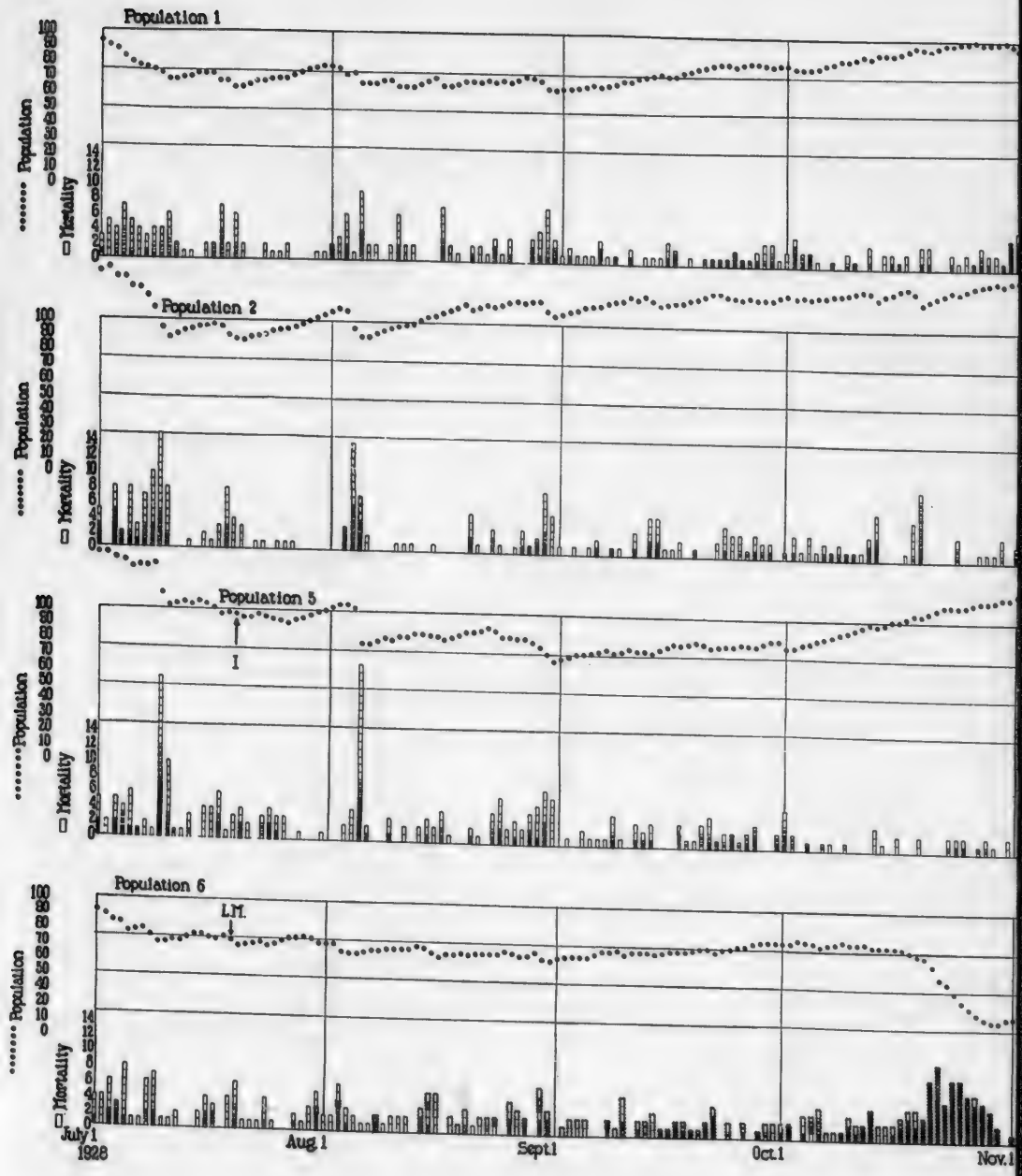
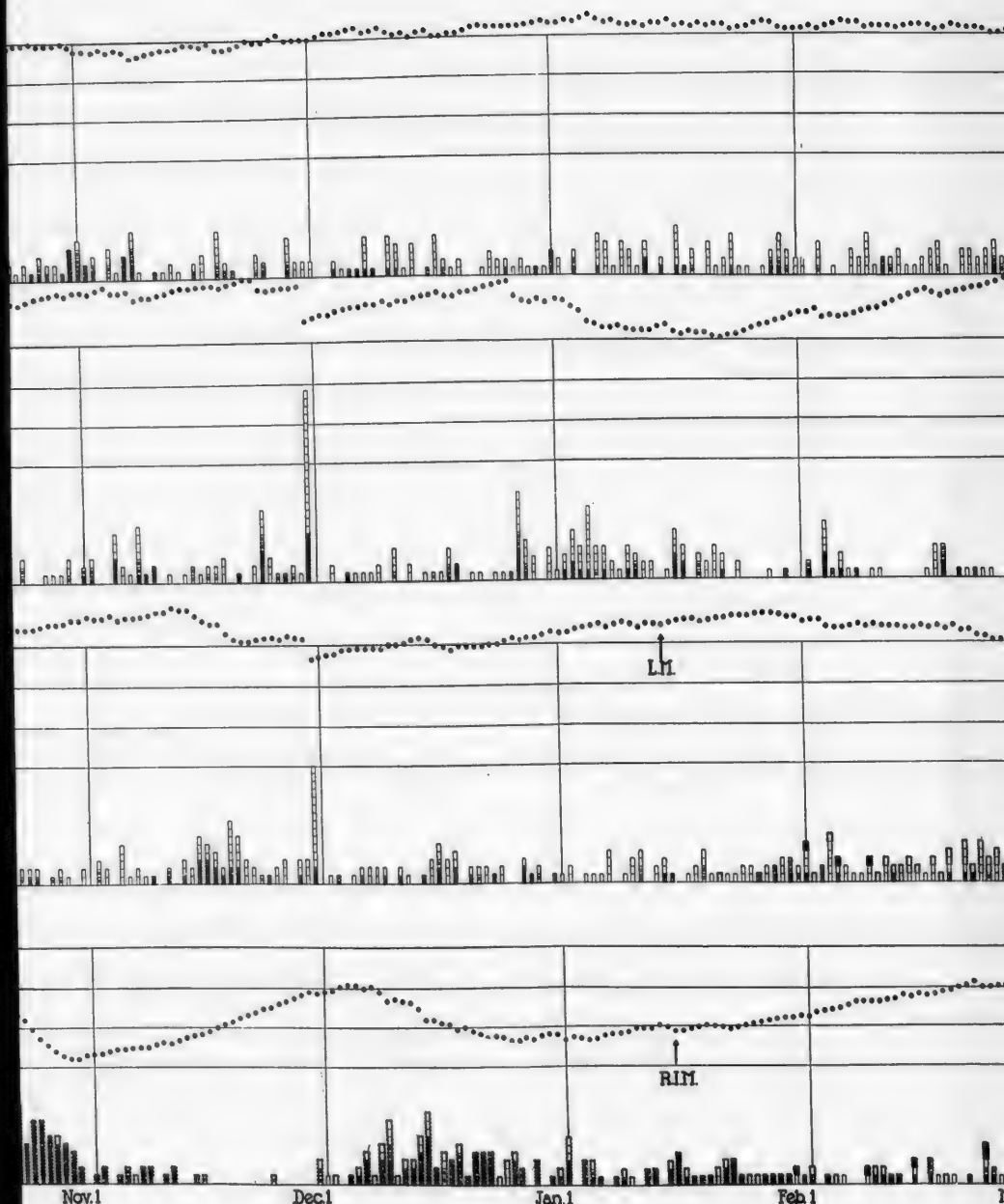
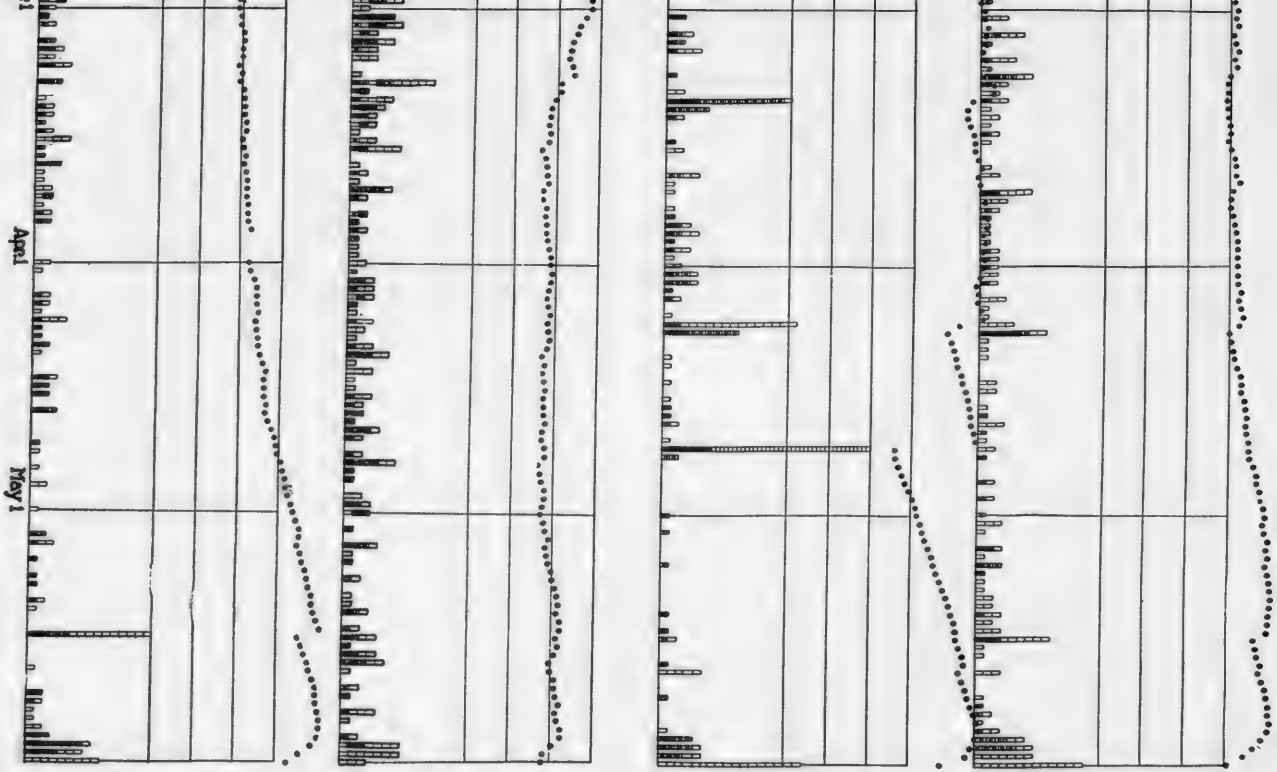

Population 1
Population
Mortality
Population 2
Population
Mortality
Population 5
I
Population
Mortality
Population 6
L.M.
Population
Mortality
July 1
1928
Aug. 1
Sept. 1
Oct. 1
Nov. 1



TEXT-FIG. 1*c*



□ - 1 mouse dead, no autopsy
◉ - 1 " " , autopsy findings negative
■ - 1 " " " " B enteritidis
□ - Lathrop mouse
? - No mice added

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populations with highest censuses. Such in brief was the characteristic behavior of the *B. enteritidis* infection in the various populations under observation.

These observations suggest that under the given conditions, microbe and host potentialities in the communities tended to establish an equilibrium, but that this equilibrium was disturbed by two influences. The explosive epidemiform waves were attributed to sudden changes in environment; the other disturbance in equilibrium evidenced by

TABLE II

Epidemiform Outbreaks of B. enteritidis Infection Associated with Changes of Environment

Population identity	Dates of increased mortality	Duration of increased mortality in days	Accompanying environmental changes
No. 1	3/8/26–3/9/26	1	Technical—Air deficiency
2	4/23/28–4/25/28	2	" Water deficiency
2	12/27/28–1/22/29	26	" " "
6	12/12/27–12/13/27	1	" " "
5	12/4/27–12/11/27	7	" " "
2	10/18/28–10/19/28	1	" Excess artificial heat
2	11/30/28	1	" " " "
2	3/12/29–3/13/29	1	" " " "
5	1/23/28–1/24/28	1	" " " "
5	5/31/28	1	" " sunlight
All	5/20/27–5/23/27	3	Hot spell
"	6/15/27–8/1/27	—	Summer heat
"	5/27/28–5/31/28	4	Hot spell
"	6/13/28–6/15/28	2	" "
"	6/27/28–7/10/28	—	" "
"	8/1/28–8/6/28	5	" "
"	8/20/28–8/31/28	11	" "

the minor wave-like fluctuations in mortality was regarded as the result of the daily immigrations of susceptible mice.[1] Finally, these observations suggested that both disturbing influences, environmental and immigrational, operated on the host rather than the microbe variable. Further evidence for this point of view is brought forward in the following pages.

[1] Topley has shown that the frequency and character of such mortality waves are associated directly with the rate of immigration of fresh mice (*J. Hyg.*, 1925, **24**, 45).

Bacteriophage and the Spread of B. enteritidis Infection

It was noted early that the *B. enteritidis* infection was accompanied by the presence of a bacteriophage. The lytic substance was demonstrable on agar plate cultures of intestinal contents from living mice (1 c), as well as on similar cultures from blood and organs obtained at autopsy. On many culture plates, colonies of *B. enteritidis* appeared in characteristic "eaten" forms and readily yielded bacteriophage effective in dilutions of 10^{-9}. The "eaten" colonies were present in cultures from intestinal tract, heart's blood, lungs, spleen, and liver, but varied to such an extent that no consistency or time relationship could be established with reference to their amount or distribution in tissues, individual mice, or mouse populations. In so far as could be determined, the bacteriophage phenomenon played no part in influencing the amount or severity of the disease.[2]

Bacterial-Dissociation Phenomenon and the Spread of B. enteritidis Infection

Observations were made on the presence of different colony types of cultures from living and dead mice of the several populations. The results of these observations have been presented elsewhere (1 c) but may be summarized as follows. From cases of spontaneous infection, smooth colony types alone were found; variants occurred only under certain definite laboratory conditions. These variants reverted regularly to the parent smooth forms, and when reverted, were similar to each other and to the naturally occurring smooth strains. No evidence, however, was obtained indicating that the phenomenon of bacterial variation occurred under natural conditions in such a way as to influence the course of individual or population disease.

A Comparison of Virulence of Pre-Epidemic, Epidemic, and Inter-Epidemic Strains of 1925–1929

At various periods during the spread of *B. enteritidis* infection the behavior of the virulence factor was tested by recovering strains of the organisms and titrating their virulence or disease-producing power. In all, two so-called pre-epidemic strains, five epidemic, and eleven

[2] Topley, studying the therapeutic value of bacteriophage in *B. aertrycke* mouse typhoid, reached a similar conclusion (*J. Hyg.*, 1925, **24**, 17).

inter-epidemic strains were tested and compared; the comparisons were made by means of a standardized virulence titration in which 20 to 50 mice were given *per os* a definite number of organisms of each of the specified strains. Details of the method have been given elsewhere (1 d): here it will suffice to describe each strain briefly and the special purpose of each titration (Table III).

Titrations 1 to 5, 1922 to 1925. The first 5 titrations, although not made with strains from these populations, are included to demonstrate certain virulence properties of *B. enteritidis*. Titrations 1 to 3 were carried out in 1922–23 with Strain M.T. 1, obtained from a mouse in the Institute, dying of spontaneous typhoid (1 e). The titrations were made to determine the effect of animal passage on virulence. The mortalities were 85, 85, and 90 per cent respectively in successive passages, indicating that the animal passage had not changed the disease-producing power of Strain M.T. 1. Titrations 4 to 5, carried out in 1925, indicated that cultivation of the Strain M.T. 1 on artificial media had also not affected its disease-producing power. These tests resulted in final mortalities of 82, 95, 95, 90 and 80 per cent respectively (1 f).

Titrations 6 to 8, 1925 to 1926, were made with 2 strains, SS_1 and SS_2, from the heart's blood of two mice in Population 2, dying Dec. 5, 1925, immediately preceding a Friedländer's bacillus outbreak (1 b). The tests were made to compare the virulence of native smooth colony cultures and reverted smooth colony forms from artificially induced variants (1 f). The mortality—95, 90, 85, 85, 95, 85, 100, and 90 per cent respectively—indicates that the native and reverted forms were equally pathogenic.

Titration 9, Oct. 3, 1927, was made to test the virulence of a post-epidemic strain, No. 1, obtained from the heart's blood of a mouse of Population 1, dying Sept. 30, 1927, following an epidemic outbreak. This outbreak was induced experimentally (page 943). The mortality was 70 per cent at 20 days, and the estimated mortality at 60 days on the basis of the standard control curve averages 80 ± per cent (1 f).

Titration 10, Jan. 24, 1928, was carried out to test the virulence of an epidemic strain, No. 2, from the heart's blood of a mouse in Population 5, dying Dec. 5, 1927. Immediately prior and subsequent to this date, the population suffered a considerable increase in mortality, due to technical disturbances of temperature. The virulence test resulted in a mortality of 70 per cent at 20 days, indicating a probable 80 ± per cent at 60 days (1 f).

Titration 11, Mar. 1, 1928, was made to test the virulence of Strain 3, obtained from the heart's blood of a mouse in Population 1, dying Feb. 27, 1928, during a quiescent period of relatively constant death rate. The resulting mortality at 60 days was 86 per cent.

Titration 12, Oct. 22, 1928, was made to test the virulence of epidemic Strain 4 and endemic Strain 5. Strain 4 was obtained from the heart's blood of an in-

dividual in Population 6, dying Oct. 19, 1928. This community was in the early phase of an artificially induced (page 943), severe epidemic, fatal to 108 mice in 30 days. Strain 5, on the other hand, came from the heart's blood of a mouse in Population 2, dying Oct. 19, 1928. This community, at that time, enjoyed the highest census and the lowest death rate for a period of 2 months prior to the test (Text-fig. 1). The titration resulted in total mortalities of 72 per cent and 84 per cent respectively.

Titration 13, Oct. 26, 1928, was carried out with the epidemic Strain 4 and the endemic Strain 5, described above, plus endemic Strain 6, from a mouse in Population 5, dying Oct. 24, 1928, during an interval of low death rate and progressive increase in the total population. The resulting mortalities were 92, 80, and 76 per cent respectively.

Titration 14, Nov. 20, 1928, was made with the epidemic Strain 7 and the endemic Strain 8. Strain 7 came from the heart's blood of a mouse in Population 5, dying Nov. 17, 1928, at the onset of a severe epidemic, fatal in 9 days to 39 mice. Strain 8 came from the blood of an individual in Population 6, dying Nov. 14, 1928, at a time of minimum death rate and progressively increasing population census. The test resulted in mortalities of 90 and 100 per cent.

Titration 15, Dec. 17, 1928, was carried out with one endemic strain, No. 9, and one epidemic strain, No. 10. Strain 9 was obtained from the blood of a mouse in Population 5, dying Dec. 14, 1928, during a period of relatively constant death rate; Strain 10, on the other hand, came from the blood of a mouse in Population 6, dying Dec. 13, 1928, at the height of a 20 day epidemic, fatal to 75 mice. The final mortalities were 85 per cent and 70 per cent.

Titration 16, Jan. 9, 1929, was planned to compare the virulence of an epidemic strain, No. 11, and endemic strain, No. 12. Strain 11 came from the heart's blood of a mouse in Population 2, dying Jan. 5, 1929, at the height of a severe mortality wave, associated with a technical alteration of environment (page 933). Strain 12, on the contrary, came from the blood of a mouse in Population 6, dying Jan. 5, 1929, at the end of a severe epidemic, just as the population census commenced a long continued rise from 41 to 100 individuals, accompanied by a correspondingly low death rate. The comparative mortalities were 95 and 90 per cent.

Titration 17, May 17, 1929, was planned to compare the virulence of autopsy and carrier strains from populations in relatively healthy and endemic states. Strain 13 came from the heart's blood of a mouse in Population 2, May 15, 1929, during a period of relatively good health and low death rate; Strain 14, from the feces of a "healthy" carrier, May 13, 1929, in the same population. Strain 15 was obtained from the heart's blood of a mouse in Population 5, dying May 7, 1929, at the time of considerable endemic mortality. Strain 16 came from the feces of a "healthy" carrier, May 10, 1929, in the same population. The titration was run in the late spring, consequently the level of resistance of the test animals was relatively high and the resulting mortalities low (1 g); however, all four strains showed a similar degree of killing power—64, 64, 60, and 68 per cent respectively.

TABLE III

Virulence of B. enteritidis

Titration No.	Date	Strain	Source	Prevalence of infection	No. mice	Mortality during days after inoculation																					Total No. dead	Per cent dead
						0-2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48*	
1	Nov. 1922–Feb. 1923	M.T. 1	Wild mouse. Rockefeller Institute (1)		20			1	5	1	1	3		2		1		3											17	85
2					20				2	4	3		2		3		1	1	1									17	85
3					20			2		4	6		1	2	1		1					1						18	90
4	Oct. 1925	No. 3	Same as M.T. 1 above (2)		27			2			11	3	2	1	1		1						1					22	82
5	Nov. 1925	" "	" " " " " " (3)		20				1	9	3	3		3														19	95
"	" " "	" "	" " " " " " (3)		20				2	2	4	2	4		4		1											19	95
"	" " "	" "	" " " " " " (3)		20			2	1	3	8	2	1	1														18	90
"	" " "	" "	" " " " " " (3)		20			1	1	5	3	2		2	1		1											16	80
6	12/20/25	SS_1	Pop. 2. Autopsy 12/5/25 (4)	Pre-epidemic period (Friedländer's bacillus)	20			1	4	1	6	6			1													19	95
7	12/23/25	"	" " " 12/5/25 (5)	" "	20			1	1	2	5	3			2	1				1				1			1	18	90
"	"	SS_2	" " " 12/5/25 (5)	" "	20			1	1	7	3			2	1	1							1					17	85
8	1/13/26	SS_1	" " " 12/5/25 (6)		20			1	2	5	3	2		1	3													17	85
"	"	SS_3	From SS_1 variants (6)		20			2	6		1	7		1							2							19	95
"	"	SS_4	" " " (6)		20			1	3	6	2		2	1	1								1					17	85
"	"	SS_5	" " " (6)		20				4	3	5	1	1		1		2		1		1	1						20	100
"	"	SS_6	" " " (6)		20				3	5	2	5		1	1				1									18	90—
9	10/ 3/27	No. 1	Pop. 1. Autopsy 9/30/27	Post-epidemic period	50			6	16	10	3				Discontinued at 20 days. Total mortality = 70%													(7) 40±	80±
10	1/24/28	" 2	" 5. " 12/ 5/27	Epidemic period	50			1	4	6	9	7	5	3	Discontinued at 20 days. Total mortality = 70%													(7) 40±	80±
11	3/ 1/28	" 3	" 1. " 2/27/28	Endemic period. Constant death rate	50				1		4	7	5	5	4	3	4		3	3	2			1		1			43	86
12	10/22/28	" 4	" 6. " 10/19/28	Onset of epidemic period	25				1	1	2	2	2	2	3	1	1		2	1									18	72
"	"	" 5	" 2. " 10/19/28	Healthy period. Low death rate	25			1		2	1	3	1	2	2	6	1				2									21	84
13	10/26/28	" 4	See above	See above	25			1	3	2	6	1	2	3					1		2		1	1					23	92—
"	"	" 5	" "	" "	25				3	1	2	3	2	1	1	1	1	1	1	1	1			1					20	80
"	"	" 6	Pop. 5. Autopsy 10/24/28	Healthy period. Low death rate	25			1	3			2	3	1	2		1		1	1	2	1	1							19	76—
14	11/20/28	" 7	" 5. " 11/17/28	Onset of epidemic period	20				2	3	2	2	1		1	2		1	1		1			1					18	90
"	"	" 8	" 6. " 11/14/28	Healthy period. Low death rate	20			2	1	1	3	5	3	1	1	2					1								20	100
15	12/17/28	" 9	" 5. " 12/14/28	" " " " "	20					4	6	3	1						1	1					1				17	85
"	"	" 10	" 6. " 12/13/28	Height of epidemic period	20				1	2	3	3		1	1	1	1		1										14	70
16	1/ 9/29	" 11	" 2. " 1/ 5/29	" " " "	20			1	2	1	1	1	3	1	1		3		1	1			1	1	1				19	95
"	"	" 12	" 6. " 1/ 5/29	Healthy period. Low death rate	20					4	6	2	1		2	1	1						1						18	90
17	5/17/29	" 13	" 2. " 5/15/29	" " " " "	25				3		4		2	1		1	1	1		2	1								16	64
"	"	" 14	" 2. Healthy carrier 5/13/29	" " " " "	25				2	4	3				1	1	1			1	1		1			1			16	64
"	"	" 15	Pop. 5. Autopsy 5/7/29	Endemic period. Constant death rate	25		1		1	3	2	3	2						2						1				15	60
"	"	" 16	" 5. Healthy carrier 5/10/29	Endemic period. Constant death rate	25			1	1	3	1	1	2				1	3					1	1		2			17	68

* No further deaths to 60 days; experiment discontinued.

(1) See *Jour. Exp. Med.*, 1923, **38**, 45, Text-fig. 1.
(2) " " " " 1928, **46**, 887, " 1.
(3) " " " " 1927, **46**, 887, " 2.
(4) See *Jour. Exp. Med.*, 1927, **46**, 887, Text-fig. 4.
(5) " " " " 1927, **46**, 887, " 5.
(6) " " " " 1927, **46**, 887, " 7.
(7) Based on expectancy. See "standard curve," *Jour. Exp. Med.*, 1927, **46**, 887.

The combined results of the titrations, shown in Table III, indicate that under natural conditions, the virulence of *B. enteritidis* is not altered by animal passage, and that "reverted" variants and strains obtained from mice are of similar pathogenicity. Finally, the tests indicate that the virulence of the organisms during pre-epidemic, epidemic, and inter-epidemic phases of the infection is relatively constant, and hence that in considering the mechanism of epidemic outbreaks of this disease the virulence factor may be regarded as unchanging.

Dosage of B. enteritidis

The second epidemic factor investigated was that of dosage, or the numbers of *B. enteritidis* available to the population at various epidemiological periods. The investigations took the form of carrier rate determinations and counts of the actual number of *B. enteritidis* in the bedding on specified occasions. The technique and results of these tests were as follows:

To determine the presence of carriers, fresh intestinal contents from each mouse were emulsified in 2 cc. of broth. 0.2 cc. of this emulsion was spread over the surface of a 15 cm. brilliant green agar plate. After 24 hours' incubation, suspected colonies were transferred to broth, incubated overnight, and tested with *B. enteritidis* agglutinating serum. The counts of *B. enteritidis* bacilli in the bedding were made by mixing the wood shavings with 3000 cc. of a 0.85 per cent sodium chloride solution, taking six 1 cc. samples of this suspension and plating 0.5 cc. of two dilutions on duplicate brilliant green agar 15 cm. plates. Thus, 24 plates were made from each lot of bedding, insuring relatively accurate counts.

Carrier determinations were made in Populations 2, 5, and 6 and total counts in Population 6. The results of these tests were as follows. In Population 5 (Text-fig. 1), carrier tests were made from Nov. 1, 1927 to Apr. 1, 1928. Prior to the first epidemic of Dec. 4, 1927, two tests were made—Nov. 28 and 30, 1927, showing 16 and 19 per cent carriers respectively. 2 and 5 days after the onset of the epidemic, the carrier rate increased to 24 and 24 per cent; at the end of the outbreak, Dec. 12 and 14, 1927, the rate fell to 13 and 16 per cent; prior to the second outbreak of Jan. 23, 1928, the rate again increased progressively—Dec. 28, 1927, 17 per cent; Jan. 4, 1928, 22 per cent; Jan. 12, 1928, 25 per cent. Later, during a period of low mortality and rising census, the rates were low—Feb. 15 and 28, 8.5 and 9 per cent respectively. In Population 2 (Text-fig. 1), carrier tests were made from Dec. 1, 1927 to Mar. 1, 1928. Prior to a period of increased mortality, commencing Feb. 1, the rate increased progressively—Jan. 9, 8 per cent; Jan. 16, 9 per cent; Jan. 30, 21 per cent. On the second day of the epidemic, however,

Feb. 3, the rate fell to 13 per cent. In Population 6 (Text-fig. 1) the carrier rate was low, 2.5 per cent on Oct. 4, 1928. On Oct. 17, however, at the onset of a severe outbreak, the rate was 57 per cent. Later, as the wave subsided, Oct. 29, the rate fell to 14 per cent, only to rise to 22 per cent on Nov. 2, before a slight secondary wave, and subsequently fall to 0 per cent as the mortality ceased. Later both carrier tests and bedding counts were made in Population 6 from Dec. 15, 1928 to June 1, 1929 (Text-fig. 1). During this period no true epidemics occurred; indeed, death rates, carrier rates, and bedding counts varied but little. The following tendencies were noteworthy, however. On Dec. 26 and Jan. 1, carrier rates were low; on Jan. 4 and 7, bedding counts were low; and from Jan. 4 to 13, the mortality rate was correspondingly low. On Jan. 7 and 11, sharp rises occurred in carrier and bedding rates respectively; a few days later, Jan. 14, a corresponding increase in mortality occurred. Again on Jan. 17, a sudden drop in carrier rate was noted; 6 days later, the mortality rate fell suddenly. A third rise in death rate occurred Feb. 22, preceded 14 days by a sharp increase in carrier rate and bedding count. Finally, during a subsequent 6 weeks' period of exceptionally low mortality, the carrier rates and bedding counts were at a corresponding minimum.

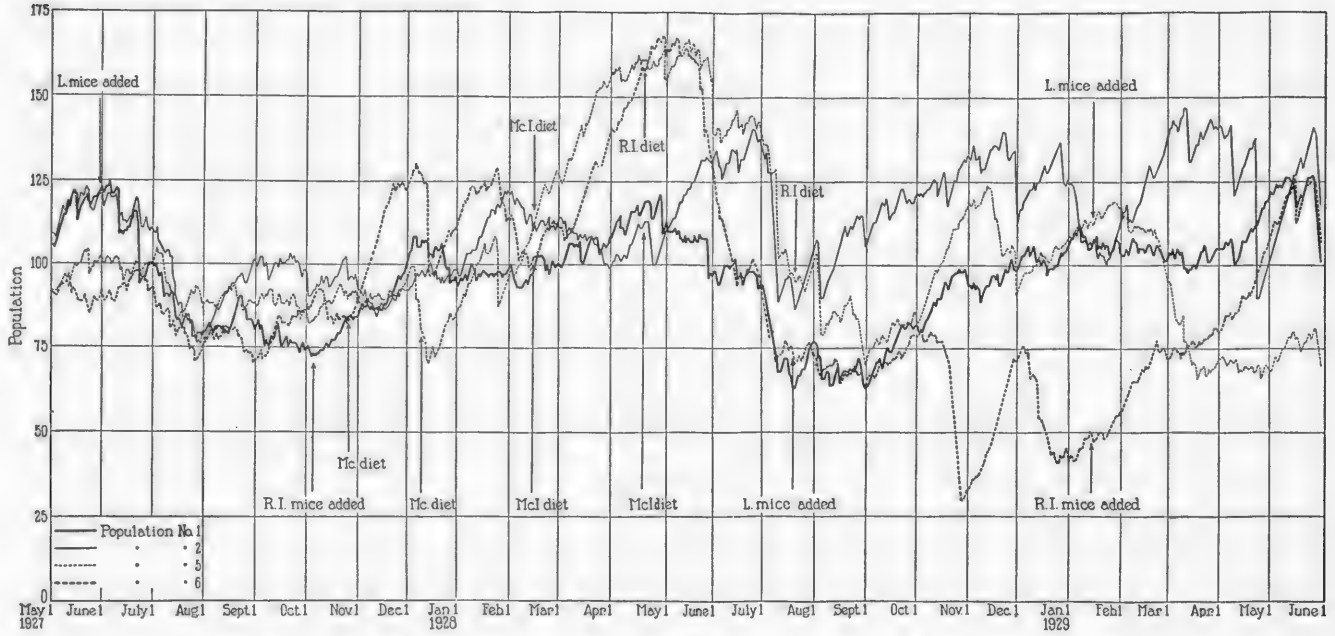
The results of the studies on the dosage factor were often marred by technical difficulties, but when conditions were favorable they indicated that dosage varied directly with mortality at a time difference approximating that of the incubation period of the disease.

Host Resistance to B. enteritidis

The behavior of the host-resistance factor in the populations was studied indirectly by altering the resistance of the populations experimentally and observing the effect of this procedure on the amount of severity of the *B. enteritidis* infection. The experimental alteration of host-resistance in the populations was carried out in two ways,—first, by modifying the routine diet, and second, by substituting in the populations immigrants of different known resistances. The results of these experiments are given in detail (Text-figs. 1 and 2).

Experiment 1.—The Effect of Modifying Host Resistance by Dietary Procedures.—The standard diet administered daily to the various populations consisted of baker's bread plus pasteurized Grade B milk. The test modification in diet was effected by replacing this ration with the standard McCollum diet.[3] This mixture had been tested pre-

[3] McCollum diet is prepared as follows: Wheat germ, 67.5 per cent; casein, 15 per cent; milk powder, 10 per cent; NaCl, 1 per cent; $CaCO_3$, 1.5 per cent; butter fat, 5 per cent.



TEXT-FIG. 2. The effect of modifying host resistance on the prevalence of *B. enteritidis* infection.

viously and found to increase the resistance of Rockefeller Institute mice to *B. aertrycke* typhoid (1 h). Similar unpublished tests showed that McCollum diet increased the resistance of Rockefeller Institute mice to *B. enteritidis* and Friedländer's bacillus infections. Consequently, these two tested diets; one, the standard bread and milk ration, resulting in relatively susceptible mice, and the other, the standard McCollum ration, resulting in relatively resistant mice, were used alternately and their effects on the spread of *B. enteritidis* infection compared.

On Oct. 26, 1927, with the census levels of Populations 1, 2, 5 and 6 at 84, 95, 91 and 83 respectively (Text-figs. 1 and 2), the diet in Population 6 was changed from bread and milk to the McCollum ration. The result was immediate. Daily mortality fell abruptly and the census rose in 26 days to 129 individuals, while the counts in the control bread and milk diet Communities 1, 2, and 5, remained at about 90. 20 days later, however, Dec. 5, the test McCollum diet community, No. 6, suffered a severe epidemic, fatal to 52 mice in 9 days, and reducing its census from 130 to 96. Subsequently, the death rate fell and the census rose rapidly to 129; but on Jan. 21, 1928, a second outbreak occurred similar to the first, save that the subsequent rise in census was less pronounced—mortality rate and census level remaining definitely within the zone of the control bread and milk diet communities. Subsequently, a similar test was made with Population 5. This community had suffered a high mortality on Dec. 4, 1927, due to a technical error, consequently its census level had dropped from 99, the level of the other control communities, No. 1 and 2, to 70. On Dec. 9, 1927, the diet was changed from bread and milk to the McCollum ration. At once its death rate fell and the census rose steadily to 108 on Jan. 20, 1928, a figure midway between the levels of the two control communities—Population 1, 96; Population 2, 115. On this date, however, an outbreak occurred reducing its census level to 87. Subsequently, the death rate fell and the census rose steadily to 111 on Feb. 13, 1928.

The results described show that an increase of population resistance by dietary procedures was highly beneficial to the communities for short intervals, but that these benefits were counteracted by the occurrence of epidemics which left the survivors in the same status as those in communities with poorer rations. Further analysis indicated that the occurrence of epidemics associated with the change in ration was probably due to the introduction into the resistant communities maintained on the McCollum diet of relatively susceptible immigrants, fed on bread and milk. Indeed, these immigrants according to survival time records contributed more than 75 per cent of the mortality during the outbreaks. Hence the technique was modified so as to increase the resistance of prospective immigrants to the McCollum diet populations by feeding them the McCollum diet 7 to 14 days before their actual admission, while the prospective immigrants to the bread and milk ration communities were kept on the bread and milk diet.

The experiment was continued on Feb. 13, 1928, by adding to the McCollum diet Populations 5 and 6 McCollum-fed immigrants. The effect of this procedure was an immediate decline in the death rate and a progressive increase in the censuses to 165 and 169 on April 30, 1928 (Text-figs. 1 and 2). Thereafter both communities maintained this relatively high level, while the two control bread and milk diet communities, Nos. 1 and 2, continued the usual level of 100 to 115. The final test consisted in substituting McCollum diet in one of the two remaining control bread and milk diet communities and its immigrants, No. 2, and replacing the McCollum diet in Population 6 and its immigrants with the standard bread and milk ration. This plan designated Population 5 as a McCollum diet control, and Population 1 as a bread and milk diet control, and tested the effect of simultaneous McCollum to bread and milk diet and bread and milk to McCollum diet substitutions in Communities 6 and 2 respectively. The consequence of this change was indeed striking (Text-fig. 2). Population 6, changed from McCollum to bread and milk diet, maintained its high census level for about 3 weeks; then by a long and severe epidemic, fatal to 150 mice in 38 days, was decimated permanently to the population level of the control bread and milk Community 1. Population 2, changed from bread and milk to McCollum rations, after a temporary rise in mortality associated with difficulties in the management of the water supply, responded in a typical manner. The death rate fell abruptly and the census level rose until on June 1, 1928, it paralleled that of the control McCollum diet community, No. 5. Subsequently, this relative 50 per cent increase in census level in the two McCollum diet communities was maintained in spite of severe weather. During the hot weather, June 25, 1928 to July 1, 1928, all populations suffered, but the communities on identical diets suffered equally and the approximate 50 per cent difference in population level between McCollum and bread and milk diet communities was maintained. One further substitution was made July 19, 1928 in Population 5, by changing its diet from McCollum to bread and milk. Two epidemic waves ensued, reducing the census of the community to that of the control bread and milk populations, Nos. 1 and 6. Meanwhile, Population 2, maintained on McCollum diet, continued its equilibrium in spite of periods of high mortality from overcrowding, at a census level approximately 50 per cent higher than that of populations on bread and milk.

These tests demonstrated that by varying an adequate diet from barely essential to more optimum constituents and *vice versa*, it was possible to incite epidemics or periods of relatively good health. Hence, since it is known that these dietary regulations result in an increase or decrease of host resistance, it is concluded that under the given conditions the level of the host resistance factor determined the degree of prevalence of the *B. enteritidis* infection.

Experiment 2.—The Effect of Modifying Host Resistance by Substituting Immigrants of Different Known Resistance.—The technique of this experiment consisted in replacing the daily quota of Institute mice immigrants for the more susceptible Lathrop mice (1 b), and *vice versa*. A substitution of Lathrop mice in a given population of Institute mice was calculated to decrease progressively the total amount of host resistance in that population; and conversely, the substitution of Institute mice in a given population of Lathrop mice was calculated to increase progressively the total resistance. These replacements were carried out in a series of tests under controlled conditions with the following results (Text-figs. 1 and 2).

Test 1.—The first substitution was made in Population 1 on May 31, 1927 (Text-figs. 1 and 2). The census in this community was 121, and in the three control communities, 121 in Population 2, 102 in Population 5, and 92 in Population 6. The replacement of Lathrop for Institute mice in Population 1 was followed by an increase in mortality which in 3 weeks reduced the census below the level of the two control communities, Nos. 2 and 5. Meanwhile the third control community, No. 6, had suffered the effects of a technical mismanagement of temperature conditions which was not corrected until Aug. 23, 1927. Shortly thereafter, its census increased to that of the control communities, Nos. 2 and 5, while the Lathrop Population 1 remained at the lowest level. Oct. 6, 1927, a reverse substitution was made in the Lathrop Population 1 by changing the daily immigrants from Lathrop to Institute mice. The census at this time of Population 1 was 73, while those of the control populations were 92 in Population 2, 84 in Population 5, and 91 in Population 6. Following the replacement of Institute immigrants in Population 1, mortality decreased and in 20 days, the census had risen to that of the control communities, after which all censuses remained alike.

Test 2.—The second substitution was made in Population 6 July 19, 1928. The census in this community was 74; in the control Population 1, it was 63, in Population 5, changed on the same day from McCollum to bread and milk diet (page 942), it was 97, and in the McCollum diet population, 88. Following the replacement of Lathrop for Institute mice in Population 6, the census level continued to parallel that of control Community 1, and after Sept. 1, control Community 5. On Oct. 3, however, with the census levels of the test and control communities at about 80 and the McCollum Community 2 at 120, the test Lathrop Population 6 suffered a severe epidemic fatal to 108 mice in 30 days and reducing the census to 29. During this period, the control communities experienced a relatively low mortality and a general rise in census levels. Virulence titrations, Nos. 12 and 13, of an epidemic strain from Population 6 and endemic strains from the healthy McCollum Population 2 and control Population 5 showed no significant differences in the pathogenicity of *B. enteritidis*. Subsequently, the test Lathrop Population

6 showed a low death date for 4 weeks during which it increased to 76 mice. A virulence titration, No. 14, made at this time, proved the infecting strain to be of full virulence. On Dec. 5, however, a second outbreak occurred, fatal to 75 mice in 20 days and reducing the census to 41. A virulence titration, No. 15, showed no differences in pathogenicity of an epidemic strain from Population 6 and an endemic strain from the control Population 5. Meanwhile, control Communities 1 and 5 maintained a census level of 100, and the McCollum community, No. 2, a level of 125.

Test 3.—The third substitutions were made in Populations 6 and 5 on Jan. 14, 1929. At this time, the Lathrop Population 6, with a census of 47, was changed by adding Institute immigrants in place of Lathrop mice, and control Population 5, with a census of 112, was changed by adding Lathrop immigrants in place of Institute mice. Population 1, with a census of 107, continued to serve as a control; Population 2, fed on McCollum diet, continued to maintain its relatively high census level. The effect of changing Lathrop to Institute immigrants in Population 6 was immediate. The death rate declined and the census level rose progressively and with little interruption until it equalled that of control Population 1 on May 14, at 125, and paralleled it closely thereafter. The contrary effect of changing Institute immigrants to Lathrop mice in Population 5 was observed. In this case the death rate increased slowly and the census fell, until on Feb. 20 a severe outbreak occurred, fatal to 97 mice in 26 days and reducing the census level on Mar. 18 to 66. Thereafter the census remained at the relatively low level of 65 to 75, while those of the two control communities, Nos. 1 and 6, ranged from 100 to 125. On June 1 the experiment was terminated.

These tests constituted a further demonstration that the amount and severity of *B. enteritidis* infection could be influenced by the experimental alteration of population resistance. When the daily immigrants to a given population were changed from a relatively resistant to a susceptible race of mice, and *vice versa*, epidemics were incited or periods of general good health brought about. Thus, under the given conditions, the host resistance factor determined the degree of prevalence of *B. enteritidis* infection.

Experiment 3.—At the end of the period of observation, June, 1929, a titration was made to determine the actual resistance to *B. enteritidis* of the survivors of the different populations.

Seventy-seven survivors of the resistant McCollum diet Population 2 were tested, 67 of the control Population 1, 75 of control Population 6, and 42 of the susceptible Lathrop Population 5, of which 24 individuals were Lathrop mice and 18 Rockefeller Institute mice. On June 18, all were given *per os* 0.5 cc. of an 18-hour broth culture of a fresh strain of *B. enteritidis* obtained from the blood of a

mouse in Population 5, found dead May 7. Each animal was then placed in a separate jar and fed on its accustomed diet. Dead animals were examined for *B. enteritidis*. The results are given in Table IV.

According to expectation, the titrated resistances of the different populations agreed with the theoretical resistances (Table IV), being greatest in the McCollum diet Population 2, similar and intermediate in the control Populations 1 and 6, and least in the Lathrop Population 5. Thus, 67 per cent of the McCollum Population 6 succumbed, 75 per cent and 77 per cent of the control Populations 1 and 6, and 81 per cent of the Lathrop Population 5, of which 100 per cent were

TABLE IV

Resistance of Population Survivors to B. enteritidis

Population No.	No. of mice injected	Mortality during days after inoculation															Total No. dead	Per cent dead
		0-2	4	6	8	10	12	14	16	18	20	22	24	26	28	30*		
1	67	3	2	4	4	5	9	5	7	4	2		1	2	1	1	50	75
2	77	2			5	10	6	4	3	2							32	41
5 R. I. (1)	18	1		1				1	1	2	2		1	1			10	55
5 Lath. (2)	24		1	2	9	6	4	1	1								24	100
5 Total	42	1	1	3	9	6	4	2	2	2	2		1	1			34	81
6	75	2	2	1	5	6	9	15	3	8		1	1	2	2	2	59	78

* No further deaths to 36 days; experiment discontinued.

(1) Rockefeller Institute mice.

(2) Lathrop mice.

Lathrops and 55 per cent Institute mice. These results furnished additional evidence that the resistance of a population could be altered experimentally by changing its diet and its racial constituents.

DISCUSSION

The present findings on the mode of spread of *B. enteritidis* infection in mouse populations may be summarized as follows. In the first place, *enteritidis* bacilli gaining access to previously unexposed communities proved to be readily infective and moderately lethal, and

when established in several populations retained these characteristics without modification. Cultures of *B. enteritidis* taken from living carriers or mice dead of typhoid during pre-epidemic, epidemic, and inter-epidemic phases of infection were of uniform pathogenicity. Furthermore, the bacterial dissociation and bacteriophage phenomena appeared to play no significant rôle in determining the spread of the infection. In the next place, the dosage or quantity of *B. enteritidis* available to population constituents, as measured by number of carriers or amount of *B. enteritidis* in the bedding, varied directly with the death rate at a time difference approximating that of the incubation period of the disease. Most interesting, however, were the experiments to test the effect of modifying host resistance on the behavior of the infection. Host resistance in the communities was raised or lowered experimentally by two proved methods—changing the diet from adequate to optimum constituents and *vice versa* (1 h) and substituting immigrants of relatively high or low racial resistance (1 b). Under the specified conditions, a depression of population resistance brought about epidemic outbreaks, whereas an enhancement of population resistance inaugurated periods of relatively good health. It is concluded, therefore, that the spread of *B. enteritidis* infection in the given populations was determined by variations in the host resistance and microbic-dosage factors in connection with the relatively constant microbic-virulence factor.

The present findings are in agreement with the previous work on mouse aertrycke (1 i), rabbit Pasteurella (1 j), and fowl Pasteurella infections (1 k). In each instance the epidemic factors were found to play a like rôle—virulence being constant, and dosage and host resistance varying and controlling the prevalence of infection. Thus, virulence, defined as microbic pathogenicity under natural conditions, was found, in spite of strain differences and variations under artificial conditions, to maintain under natural conditions an average constant level. Besides, virulence appeared related to infectivity or vegetative capacity in an inverse manner—the greater the lethal potency, the less the ability to remain established in a given community. Dosage, on the contrary, proved to be a varying factor, increasing prior to an outbreak and decreasing shortly before the end of an epidemic. Furthermore, a suitable dose of microbes administered to a group of

animals was capable of inciting an epidemic similar in every way to those occurring "spontaneously" under natural conditions. Finally, host resistance was found to be a varying factor, possessing racial, individual, and specific components, the total amount of which controlled the severity of infection both in the individual and in the herd.

With these facts as a basis, the epidemiology of six native animal infectious diseases may be described as follows. Etiologically, epidemics were of three general varieties: (a) those occurring when a virulent strain of bacteria gained access to a previously unexposed population; (b) those occurring in infected communities, when dosage was increased by some environmental agent; and (c) those occurring in infected communities, when population resistance was lowered. In the first instance, which involved the access of foreign microbes to a previously unexposed community, the sequence of events depended primarily on the balance between the inherent virulence of the microbes and the resistance of the population. Highly virulent microbes in a community with average resistance induced explosive, fatal epidemics and tended to disappear; the less virulent forms gave rise to moderate outbreaks and did or did not become permanently established; the least virulent forms gained no foothold. The level of total population resistance detracted from or added to the apparent virulence of the microbes. In the second category, in which epidemics occurred in an infected community when dosage was increased by an environmental agent, such as water, milk, food, housing, etc., the sequence of events in a community with average resistance depended primarily on the inherent virulence and the available dosage of microbes. Large doses of microbes of average virulence induced explosive epidemics, while smaller doses gave rise to more moderate outbreaks. In the third category, in which epidemics occurred in an infected community, when resistance was decreased by immigration, food, season, and other unknown influences, the sequence of events was a fall in resistance to a critical level followed by an increase in dosage and increase in disease prevalence. In each instance, individual differences in resistance of the population constituents determined the form of the epidemic curve, and the numbers of susceptible individuals determined the duration of the epidemic. Inherent resistance formed the basis of host resistance; specific immunity in these

relatively acute and fatal infections played a minor though significant rôle. In short, the spread of these six native animal diseases seemed to be controlled not by fluctuations in microbic virulence but by definite variations in the resistance and dosage factors in connection with the relatively fixed virulence of the microorganism.

SUMMARY

1. Several mouse populations under experimental observation were infected with *B. enteritidis* typhoid. The infection took the form of periods of low grade mortality interspersed with epidemiform outbreaks. During the periods of low grade mortality, the number of daily deaths was either relatively constant or rhythmic in 9 day intervals. The epidemiform outbreaks were associated invariably with some definite environmental disturbance.

2. The spread of this infection was not influenced by the presence of bacteriophage or the bacterial-dissociation phenomena.

3. The virulence or disease-producing power of *B. enteritidis* was found to be constant during all pre-epidemic, epidemic, and endemic phases of the infection.

4. The dosage of *B. enteritidis* available to a population at a given time was found to vary directly with mortality at a time difference approximating that of the incubation period of the disease.

5. The amount and severity of *B. enteritidis* infection in the populations were controlled experimentally by varying the diet from adequate to optimum constituents and by varying the daily immigrants from a relatively resistant to a susceptible race of mice.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES

XII. The Fixation for Histological Purposes of Erythrolitmin after Vital Staining, with a Note on the Distribution of the Indicator

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(Received for publication, August 27, 1930)

Erythrolitmin possesses singular potentialities as a vital stain because it continues to behave as an indicator during the many weeks that it remains in the body. McMaster has utilized it for a study of renal physiology (1). To increase the usefulness of the dyestuff a method is desirable for its preservation *in situ* within tissues fixed for histological purposes. The following procedure has proven satisfactory.

The animals are stained with a 2 per cent solution of purified erythrolitmin (2), made up just prior to use and boiled for 2 minutes to sterilize it. Four intraperitoneal injections of 0.5 cc. of the solution at intervals of 2 days result in a marked and widespread vital staining of adult mice. The animals are killed 2 days after the last injection. For rats 1 cc. is used. With rabbits the route of administration should be varied, four injections of 5 cc. being given at intervals of 3 days, the first intravenous, the next intraperitoneal, then one into the subcutaneous tissue of the flank, and finally an intraperitoneal injection again. The situation of the dye in the tissues varies with the interval elapsing after its introduction (3).

As fixative 8 per cent formalin in isotonic saline solution is employed (20 cc. of commercial formalin solution to 80 cc. of 0.9 per cent salt solution). The slices of tissue should be removed with a sharp scalpel or razor, since scissors cause much local damage as disclosed by changes in the indicator color. The material may be left in the formalin solution for a long period without harm, but 24 hours is sufficient. Dehydration, clearing and embedding in paraffin are done as ordinarily. The sections are lightly stained with hematoxylin (Ehrlich's or Delafield's), differentiated with acid alcohol, transferred to tap water for 2 minutes, dehydrated and mounted in balsam. When first mounted the erythrolitmin is blue, as result of the alkalinity of the tap water; but within 24 hours the acidity of the balsam

has sufficed to turn it red. Granules containing it are sharply demarcated and light pink to dark red, according to the amount present. The sections keep well in the dark, but strong light turns the dye brown.

Erythrolitmin is laid down in such concentration within some cell granules that when it begins to dissolve a colored cloud forms about them, blurring their margins (4). An excellent criterion is thus provided of the adequacy of various methods of fixation. The one here described retains the indicator without perceptible loss. Needless to say, however, those color nuances from red through violet to blue which are noted in the living tissues and which give to the dye its special value as an indicator of vital conditions, are wholly lost. In fixed specimens the hue alters with the intercurrent changes in reaction.

The localizations of pigment in the living tissues of animals vitally stained with litmus, as such, have already been reported upon (5). There is no need to describe comprehensively the pigmentation in specimens fixed and sectioned after staining with erythrolitmin, since it is almost identical with that after litmus injections, as would follow from the fact that the dye is not only the principal ingredient of litmus but the one having greatest tinctorial value as a vital stain. The matrix of certain tissues stains diffusely (bone, cartilage and connective tissue), appearing a brilliant blue during life. In the cells of numerous organs the dye is stored in granular form, and in all healthy cells in which this happens, except those of the upper portion of the convoluted tubules of the kidney, these granules are red. The macrophages everywhere throughout the body store erythrolitmin in large, homogeneous granules. Like other material ingested in quantity by such cells, it gives rise to proliferation of them. After an injection of litmus the staining is at first most intense near the surface of the macrophage granules (6); but later it becomes the same throughout them. The latter condition was regularly found in the fixed erythrolitmin specimens, doubtless because the animals were killed late in the process of storage. The fibroblasts everywhere contained fine granules of the stain, as Evans and Scott (7) have noted of other acid dyes, and Rous of litmus. Very occasionally a connective tissue fibre showed a few minute granules at its junction with the fibroblast.

In order to determine how widely erythrolitmin can be distributed in the tissues, mice were given repeated intraperitoneal injections of it over a period of 3 months, each animal receiving 23 cc. of 2 per cent solution in all, or a total of nearly 0.5 gm. of dye. They did not lose

weight, remaining healthy to all appearances. At autopsy the staining proved intense and widespread. The findings were in general the same as in animals heavily stained with litmus, and hence will not be gone into at length; yet certain localizations deserve record. The adipose tissue of the omentum and that around the kidneys and uterus had undergone an extraordinary change in constitution. It no longer contained fat but was now composed of large cells distended with erythrolitmin, in most cases as a solitary, large red granule with the cell nucleus at the periphery as in the ordinary "seal-ring" arrangement of the fat cell. A similar change has been reported for other highly colloidal dyes. Granular staining had taken place in many elements besides those of the reticulo-endothelial system. Some cardiac muscle fibres contained fine granules of erythrolitmin next the nuclear poles. A few of the smooth muscle fibres of the abdominal viscera generally,—with the one exception of the uterus,—likewise held them. The cells of the pancreatic acini contained a few granules showing the indicator, but the islet tissue was unstained. The vascular endothelium throughout the body, and the cells of Bowman's capsule (kidney glomeruli) exhibited a fine granular staining. The transitional epithelial cells of the bladder,—but not those of the ureter or kidney pelvis,—held a few granules. Hesse (8) has noted a similar localization of trypan blue in the bladder epithelium. A few ciliated columnar epithelial cells of the respiratory system contained fine granules. Glasunow (9) states that trypan blue stains many of these cells when repeatedly injected into guinea pigs. The skeletal muscle, the brain (including the neuroglia) and the parenchyma of the salivary glands, thymus, thyroids, and parathyroids, contained none of the indicator. The tela choroidea had many macrophages within its meshes that had stored erythrolitmin, but the secretory cells were unstained.

Mice given four injections of 0.5 cc. each hold the erythrolitmin without apparent loss for periods of 6 months or more. Rats lose a little of the dye in that time, but even after 9 months the granular and diffuse staining is well marked, a fact noted after vital staining with litmus.

DISCUSSION

The distribution and segregation of highly colloidal vital dyes follow definite lines irrespective of the pigment employed (10), the localization of one dye differing from that of another only in details (11). Erythrolitmin well exemplifies this rule. The minutiae of the staining here reported are not unique, nor indeed are they peculiar to extraneous pigments. The granular localization in cardiac muscle resembles that seen in brown atrophy of the heart, while the general distribution of erythrolitmin has similarities to that of the pigments present in clinical and experimental hemochromatosis (12). There is reason to suppose that the abundance of pigment in the liver parenchyma in this disease is the result of hepatic damage. Taken together, the facts give ground for the supposition that the morbid pigments just mentioned are segregated within the body by the same processes as are the highly colloidal "acid" dyes, a view already put forward by v. Möllendorff (13).

SUMMARY

A method for fixing erythrolitmin in tissues for subsequent histological examination is described. Some features are recorded of the distribution of the indicator in animals receiving it in large quantity.

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THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES

XIII. The Reaction Prevailing during the Autolysis in Vivo of Small Tissue Masses

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(Received for publication, August 27, 1930)

Many observers have noted that during tissue autolysis *in vitro* a slightly acid reaction prevails (1); and the recognition of this condition, so vital to the activity of certain enzymes, has led to not a few suppositions upon what takes place after necrosis within the body. Where large masses are breaking down *in vivo* the reaction is undoubtedly acid; for not only do they become so well walled off as to be little influenced by body processes, but actual determinations have been made upon them and a slight acidity detected (2). Recent work (3) indicates, however, that where cells die singly or in small aggregates, —and most necrosis is of this character,—the local reaction turns toward alkalinity, in some situations at least. The experiments here reported were undertaken to enlarge these observations, and with the further object of finding whether changes in the color of tissue elements vitally stained with indicators provide more sensitive criteria of cell life and death than do the histological alterations commonly relied upon. Advantage has been taken of a method (4) whereby erythrolitmin can be preserved *in situ* in fixed tissue.

As the result of normal cellular activity most living tissues are relatively acid as compared with the blood, not a few having a reaction well below pH 7.0 (5), while within cytoplasmic granules the acidity, as evidenced by segregated indicators, may be still more pronounced (3). When individual cell death occurs, or is induced, pronounced changes occur in the color of the indicators which point to a shift in the reaction toward alkalinity. Litmus or erythrolitmin, already segregated within the protoplasm in the red form, turns sharply blue and dissolves out in a blue cloud (3). Dying skin grafts on animals injected with phenol red do not stain red like normal skin, or ruddy orange like healthy grafts, but a somewhat

purply red, showing that they have become relatively alkaline (6). These changes are to be explained by a loss of the selective semipermeability of the cells with the consequence that the alkaline body fluids seep into them, while, in the case of skin grafts, carbon dioxide escapes through the surface epithelium. That they are actually due to such causes numerous data attest (6).

In a first series of experiments with erythrolitmin-stained animals the attempt was made to damage individual Kupffer cells and parenchymal elements of the liver without causing any massive necrosis. The specimens were studied in the fresh by a technic which experience has approved (7), and after fixation with the indicator *in situ* they were sectioned and searched for histological evidence of injury.

The Changes in Cells Killed with Streptococci

Mice stained by several intraperitoneal injections of erythrolitmin solution were used for the most part. For some hours after introduction of the indicator the blood appeared blue with it, but soon it was stored here and there in the form of bright red granules. Fresh sections of the healthy liver, made with the Valentine knife, appeared pink because of the great amount of erythrolitmin segregated within the Kupffer cells. The parenchyma contained practically none.

Death of individual cells and of cell groups was readily brought about by the intraperitoneal injection of a virulent strain of hemolytic streptococci.* It was already known that normal mice injected intraperitoneally with litmus have after 24 hours a blue peritoneal fluid in which are suspended many cells containing granules ruddy with the indicator. As time passes a certain proportion of such cells sicken and die, as shown by a change in the staining of those procured by aspiration (3). In cells which succumb the granules turn violet, then blue, and the indicator, dissolving out, stains the nucleus which until this time has been uncolored. Preliminary counts of erythrolitmin-containing cells from the peritoneal cavity showed that in my animals the number that were recognizably dead or dying,—which were, that is to say, diffusely blue or contained blue granules,—varied from 0 to 5 for every 200 cells that were living as proved by a red granulation. Within a few hours after the injection into stained mice of streptococci (0.01 cc. to 0.05 cc. of a 24 hour broth culture) the proportion of blue cells increased until after 24 hours all of the aspirated cells were blue in some instances. When after 1 to 3 days, the animals themselves appeared moribund, they were killed with chloroform and an immediate post-mortem examination done. Spreads of

*The strain of organisms was kindly provided by Dr. Todd of the Hospital of The Rockefeller Institute.

connective tissue, mesentery, omentum, and the suspensory ligament of the liver, were made on mica slides and covered immediately with mica coverslips to prevent drying. Valentine knife sections were also made of the liver and spleen and similarly mounted. Some of the post-mortems were done beneath washed liquid paraffin oil as precaution against changes in cell reaction due to exposure or drying. An attempt was made to correlate the ratio of dead to living cells in a piece of tissue or in the aspirated fluid with that of cells with or without intracellular streptococci. For this purpose the spreads and films were rapidly fixed and strained by Gram's method. Occasionally a rough relation between these ratios was observed in the aspirated specimens.

The severer the infection the greater was the proportion of "blue" cells, cells that is to say with blue granules or cytoplasm. The subcutaneous connective tissue over the abdomen which was not only traumatized by the successive aspirations but incidentally reinoculated showed many blue macrophages. Tissue of the same sort taken from the thorax or leg contained no dead cells, the granules being pink everywhere. In one or two mice all of the mesenteric macrophages were blue, together with most of those in the omentum and a lesser number in the suspensory ligaments of the liver; but in the majority of instances the cell damage was not so great. The liver usually showed no macroscopic color abnormalities, but occasionally a necrosis of entire lobuli was recognizable in the gross as a blue patch amidst the pink. Ordinarily the Valentine knife sections showed scattered blue Kupffer cells under the microscope, sometimes many of them, sometimes few. Frequently one or two blue Kupffer cells only were discoverable in a field, but once 20 of them were counted to 22 "red" cells. Of these 20 blue cells one exhibited a diffuse cytoplasmic staining. The other 19 still showed well demarcated granules, indicating that cell death was fairly recent. On fresh section the Kupffer cells within the lobular necroses were found to be deep blue, diffusely colored ordinarily, amongst lighter blue, evenly stained parenchymal cells. The spleen on section showed many dye-containing macrophages but rarely any blue ones except in the capsule.

In paraffin sections of the material containing the scattered, dead Kupffer cells fixed in 8 per cent formol saline and stained by Ehrlich's or Delafield's hematoxylin, these elements could not be distinguished from normal ones. In the rare instances in which entire lobuli had become necrotic as shown by a generalized blue staining with erythrolitmin there was on the other hand to be discerned in the sections a diffuse staining of the whole lobule and early fatty degeneration of the glandular cells. Spreads of mesentery, suspensory ligaments of liver, and connective tissue, which had shown scattered dead cells in the fresh preparations, were similarly fixed and stained. In such specimens no evidence of the existing cell injury and death could be discerned.

The experiments were repeated in rats, but the streptococci did not kill these animals and no liver lesions were induced. A significant fact developed, however. The number of blue cells in the peritoneal fluid withdrawn 24 hours after injection of the streptococci came to 16 to 90 per cent of the total, whereas preliminary

aspiration had yielded only 5 per cent at most. After 48 hours the number of blue cells had usually decreased, though in one aspiration all were blue at this time. Thereafter the proportion of them receded to that met with prior to the inoculation. None of the animals appeared ill at any time, with the exception of one which developed a scrotal abscess yielding lavender-blue pus. In the rat the peritoneal cavity opens directly into the scrotal sac and dead leucocytes from the peritoneal fluid accumulate within the sac (3).

The Changes in Cells Killed with Other Organisms

Mouse Typhoid Bacilli.—The proportion of the macrophages in the peritoneal fluid which were killed by intraperitoneal injection of suspensions of this organism was less than with streptococci, though groups of blue cells were encountered on the visceral peritoneum. Very few Kupffer cells were killed.

Pneumococci.—The findings were similar to those with mouse typhoid. The animals very soon died.

*Tubercle Bacilli (Avian).**—Mice were given 0.05 cc. of a suspension of the bacilli in saline (0.5 mg. per 1 cc. saline) intraperitoneally. They were sacrificed at intervals of 5 to 28 days after the injection. Similar results were obtained in rabbits killed 19 or 20 days after inoculation. Tubercles developed plentifully but the numerous cells containing erythrolitmin were not involved in their formation. Dr. Sabin and Dr. Doan assisted in the appraisal of the findings.

Intercurrent Infections.—In erythrolitmin-stained rats dying of "lung disease," isolated blue macrophages were present in the mediastinal pleura. No evidence of cell death was found within the peritoneum. Some animals had minute focal necroses of uncertain etiology within the liver. These attracted notice post-mortem by their blue color.

Pus from a pyothorax of a mouse was lavender-blue.

The findings prove that the invasion of streptococci by way of the abdominal cavity causes not infrequently a scattered destruction of individual Kupffer cells, as shown by a change in the color of erythrolitmin, previously stored in these elements, from red to blue with secondary diffusion of the dye. The cell death is perceptible by the indicator method before any changes have occurred that would enable one to recognize it by ordinary histological criteria. When the method was used whereby erythrolitmin is fixed *in situ* (4) the indicator was regularly found to be diffusely distributed throughout the cytoplasm of such cells as were recognizably necrotic on hematoxylin staining. Never did it have the granular localization characteristic of cells recently dead.

*Kindly provided by Dr. Doan.

Cultures of hemolytic streptococci are known to develop an acidity of pH 5 to pH 5.2 in both sugar free and sugar containing media (8, 9, 10). In view of this fact one would expect the organisms to render cells that they had killed more acid if anything. The marked change that took place in the direction of alkalinity can scarcely be laid to their growth. Nevertheless to rule out this possibility completely some attempts were made to kill scattered Kupffer cells by means of chemical agents. These were not successful.

Neither mercuric chloride, injected into the blood stream, nor benzol given subcutaneously, nor silica sol (11) administered intravenously and intraperitoneally (to rabbits as well as mice) caused recognizable injury of the Kupffer cells.

Sublethal doses of ricin (kindly provided by Dr. Landsteiner) were injected subcutaneously into mice and the animals were killed and examined 1 to 6 days afterwards. At the site of the injection were a few blue macrophages, but elsewhere in the connective tissue these elements held red granules only. In the mesentery and omentum were isolated small areas containing blue cells, either in aggregates or scattered amongst others containing red granules. The liver showed a few lobular necroses here and there, colored diffusely blue, the Kupffer cells being more deeply stained than the parenchyma. Elsewhere the liver tissue appeared healthy. No necroses were observed in the spleen.

The Changes in Damaged Skin

In some subsidiary tests small areas of skin were damaged by freezing. The changes in reaction of grafts injured by the method has already been referred to. The object of the present work was to compare the indicator changes manifested by tissue damaged *in situ* with those disclosed by ordinary histological methods.

Small areas of the shaved skin of litmus-stained mice, rats and rabbits were frozen with pencils of CO_2 snow for various periods and at various pressures. The pencil was cut from "dry ice" by means of a cork borer of approximately 3 mm. diameter for mice and 3 to 10 mm. diameter for rabbits. It was applied directly to the shaved skin, a loose-fitting metal rod inside the borer transmitting the pressure desired. The thawing of the patch was followed, sometimes with the dissecting microscope, again with the naked eye. The white disc of frozen skin was at once surrounded by a very narrow blue halo, and as thawing took place the general color of the disc was seen to have changed to deep blue in striking contrast to the pinky red of the tissue elsewhere. The patches were examined frequently thereafter. The deep blue color slowly gave place to a very pale blue, at first at the periphery and later throughout the whole patch, the eventual color

being attributable to dye retained by the connective tissue fibrils. This color remained unaltered from the 4th day after the injury for periods up to 6 months. During this long time there occurred no wandering into the patch of macrophages containing red erythrolitmin though these were present in the surrounding tissue in large number.

It was found that the application of the pencil for 3 seconds at a pressure of 10 gm. to the skin of mice was sufficient to kill the macrophages as shown by the erythrolitmin changes but that it did not permanently damage the epidermis. Usually a slight edema developed and after a day or two a fine desquamation over the area. In most experiments the hair continued to grow at the same rate as in the normal skin round about, but if the time of freezing was lengthened to 5 seconds or more it ceased to grow for a day or two. The skin of rabbits required freezing for 10 seconds with a pressure of about 50 gm. to turn the macrophage granules from red to blue. There followed local edema and scabbing which complicated the results. The erythrolitmin color changes were, however, the same as in the mice.

Specimens of the damaged skin were removed at intervals of from 15 minutes to several months after the time of freezing, sectioned in paraffin and examined after staining with hematoxylin. Even in the specimens removed after 15 minutes the cytoplasm of the macrophages could be seen to be badly damaged, but nuclear changes were not apparent until 1 hour and 15 minutes afterwards. The formation of intracellular ice is a disruptive process giving rise to clear-cut cytological changes. Hence the method proved a poor one for my purpose.

Significance of the Changes

The errors to which erythrolitmin is liable as an indicator have been analyzed and found to be large (12). In the presence of salts and protein its range is shifted toward the acid side, the result being that the change from red to blue does not mean an alteration from frank acidity to frank alkalinity but merely a diminution in acidity. This fact has been demonstrated once again in the following experiments:

The erythrolitmin-containing cells obtained by peritoneal aspiration of stained animals were separated by centrifugation from the fluid containing them and resuspended in phosphate buffer solutions of various hydrogen ion concentrations. The cells that were already blue retained their color in solutions more alkaline than pH 6.47 at which a purplish tinge was first evident. At pH 5.90 the blue cells turned red, but their color first became identical with that of erythrolitmin segregated in the living cells at pH 5.59. Pieces of liver that had been repeatedly frozen and thawed to kill the Kupffer cells were similarly treated and gave identical results.

It is plain that cells blue with erythrolitmin can be as acid as pH 6.5.

The Changes in Damaged Cartilage

The phthalein indicators phenol red and brom cresol purple give reliable evidence of the reaction within living tissues (13). They were now pressed into service in an attempt to ascertain the reaction of dead Kupffer cells. Unfortunately the staining of the individual elements proved too pale for this purpose. Clear cut results were obtained with cartilage though.

The freezing pencil was applied to the inner surface of the pinna as well as to the shaved skin of the flank of a white rabbit. Areas 6 to 10 mm. in diameter were frozen for 10 seconds or longer immediately before or after an intravenous injection of phenol red in quantity sufficient for marked general staining. The normal cartilage of the pinna becomes orange-pink by transmitted light in animals stained with this dye. That which had been frozen, on the other hand, stained a frank red and on each of 4 successive days upon which the injection of the dye was repeated it stained red again in marked contrast to the adjacent tissue of the same sort. As decolorization took place,—usually completed in about 5 hours,—the dye lingered in the damaged areas. As long as they retained it their color was distinctly red.

The dye penetrated the damaged skin of the flank less rapidly than normal tissue, but the color eventually assumed was identical with that of normal skin. Where stained skin is frozen, however, the color on thawing is at first a frank yellow, owing, no doubt, to a localized acidosis consequent on impaired circulation. After an interval of a few minutes the hue becomes identical with that elsewhere.

1 cc. of phenol red was injected intraperitoneally in mice and areas of skin 3 by 3 mm. in diameter were frozen before and after the dye injection. As in rabbits the indicator took longer to penetrate the recently thawed areas than it did normal skin. The spots were ruddy at first at the periphery but had a yellowish hue at their centers. Later, however, when the dye had penetrated the spots completely, their color by reflected light was indistinguishable from that of the surrounding skin. As decolorization took place the dye remaining in the spots assumed a reddish yellow hue at their centers, but at the periphery, where the damage was equally severe, as shown by the erythrolitmin experiments and also by the histological findings, the hue remained red so long as the staining endured.

The experiments with phenol red leave no doubt that cartilage damaged *in situ* by freezing becomes relatively alkaline as compared with the normal tissue. The color it assumed was that associated with pH 7.4. No evidence of such a change could be observed by reflected light in skin injured in the same way.

DISCUSSION

In its prime object to find whether cells dying individually within the body become frankly alkaline the work has proved inconclusive. When within the cells the range of erythrolitmin was found to be so shifted that it is blue even at pH 6.5. Erythrolitmin within injured cells assumes the color of alkalinity yet this it might do while the cell reaction was still on the acid side of neutrality. That such is the actual state of affairs in skin damaged by thawing has been demonstrated by my observations with phenol red.

The hue of injured cartilage stained with phenol red indicates a change from the normal condition of relative acidity to a reaction approximating that of the blood. The failure of skin frozen *in situ* to become ultimately more alkaline than its surroundings, on test with phenol red, like skin grafts frozen *in vitro* at the time of transfer (6), can be laid to the severity of the injury inflicted on these latter. They had been frozen and thawed three or four times in quick succession.

The comparison of fresh and fixed specimens of liver material containing injured Kupffer cells has shown that cell death is demonstrable with erythrolitmin when it cannot as yet be recognized by means of histological alterations. Doubtless in the future many indicators of cell conditions will be pressed into service for tissue study, supplementing, when they do not supplant, the crude criteria of morphological change.

SUMMARY

By a variety of methods necrosis of individual cells was produced in the living animal. Cell death was found to be clearly perceptible by means of the changes in the color of segregated erythrolitmin at a time when it was not as yet recognizable by ordinary histological methods. The hue of the dye pointed to an alteration in the direction of alkalinity; but the extent of this could not be determined owing to the limited range of the indicator

Cartilage damaged by freezing *in situ* becomes alkaline, its reaction now approximating that of the blood.

The findings support the view that within the body the autolysis of individual cells and of small tissue masses may take place under the circumstances of a slight alkalinity.

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